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# Role of Ataxia-Telangiectasia Mutated Kinase in Cardiac Autophagy and Glucose Metabolism Under Ischemic Conditions

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ABSTRACT

Role of Ataxia-Telangiectasia Mutated Kinase in Cardiac Autophagy and Glucose Metabolism Under Ischemic Conditions

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Ataxia-telangiectasia mutated kinase (ATM), a serine/threonine kinase primarily located in the nucleus, is typically activated in response to DNA damage. Individuals with mutations in *ATM* gene develop a disease called Ataxia telangiectasia (AT). These individuals are more susceptible to ischemic heart disease and metabolic disorder. Our lab has previously shown that ATM plays a critical role in β-adrenergic receptor (β-AR) - and myocardial infarction (MI)-stimulated myocyte apoptosis and cardiac remodeling. This study tested the hypothesis that ATM plays a critical role in cardiac autophagy and glucose metabolism following MI and ischemia, respectively. Early during MI (4 hours after its onset) and 4 hours post-treatment with ATM inhibitor KU-55933, ATM deficiency resulted in autophagic impairment in the heart and in cardiac fibroblasts, respectively. Such autophagic changes in the heart and in cardiac fibroblasts associated with the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK. ATM deficiency also augmented autophagy in the infarct region of the heart 28 days post-MI as well as in cardiac fibroblasts treated with ATM inhibitor KU-55933 for 24 hours. Autophagic changes in the infarct region during ATM deficiency associated with enhanced Akt, Erk1/2, and mTOR activation. Additionally, the lack of ATM accelerated glycolysis and gluconeogenesis and augmented TCA cycle metabolism under non-ischemic conditions. Following a 20 minute global ischemic period, the glycolytic pathway, not the gluconeogenic pathway, was down-regulated during ATM deficiency which was found to be associated with alterations in TCA cycle

metabolism. Such metabolic rearrangements associated with changes in the phosphorylation of Akt, GSK-3β, and AMPK alongside alterations in Glut4 protein expression. Thus, ATM deficiency impairs autophagy early after the onset of MI and in cardiac fibroblasts treated with ATM inhibitor KU-55933 for 4 hours. In contrast, ATM deficiency appears to augment autophagy late post-MI in the infarct region of the heart and in cardiac fibroblasts treated with ATM inhibitor KU-55933 for 24 hours. Lack of ATM alters glucose and TCA cycle metabolism with and without ischemia. Such findings implicate ATM as a key player in autophagic changes in the heart in response to MI as well as in glucose metabolism under non-ischemic and ischemic conditions.

# DEDICATION

To my family: I want to sincerely thank you for always being here for me and doing whatever you can to make sure that I succeed. Without you I would not be where I am today and I am forever indebted to you for that.

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# CHAPTER 1

# INTRODUCTION

## Myocardial Infarction and Heart Failure

Heart failure is the leading cause of death worldwide; in fact, 17.3 million of the 54 million total deaths in the world in 2013 were due to cardiovascular disease. Roughly 92.1 million adults in the United States have at least one type of cardiovascular disease and those numbers are expected to increase (Go et al. 2017). Heart disease can occur in response to many conditions like coronary artery disease, chronic hypertension, atherosclerosis, and myocardial infarction (MI) (Frangogiannis 2008). MI ultimately leads to heart failure by initiating a cascade of events collectively known as left ventricular remodeling (LV remodeling/cardiac remodeling), a process characterized by changes in ventricular function, shape, and size (Sutton and Sharpe 2000). LV remodeling is divided into an early phase (within 72 hours post infarction) and a late phase (beyond 72 hours post infarction) (Sutton and Sharpe 2000). Within hours of MI, cardiac myocytes (the fundamental contractile cell of the myocardium) begin to undergo apoptosis (Sutton and Sharpe 2000; Shih et al. 2010). Cardiac myocyte apoptosis triggers expansion of the infarcted area (early phase cardiac remodeling) that is subsequently followed by myocardial hypertrophy, cardiac fibrosis, and a deterioration of contractile function (late phase cardiac remodeling) (Sutton and Sharpe 2000; Shih et al. 2010). Collectively, this process results in a poorly functioning left ventricle that progresses to heart failure (Sutton and Sharpe 2000; Shih et al. 2010).

## Autophagy and Heart Failure

Autophagy is a conserved physiological process in the body that ultimately results in the packaging of damaged cytoplasmic components into autophagosomes that fuse to lysosomes for degradation (Klionsky et al. 2007; Meijer and Codogno 2009; Jimenez et al. 2014; Bravo-San Pedro et al. 2017). Autophagy consist of three main phases: 1) induction and phagophore formation, 2) phagophore elongation and autophagosome formation, and 3) lysosomal fusion, degradation, and recycling (Mizushima 2007). Autophagy is critical to cellular homeostasis both under normal and stressful conditions. Despite its beneficial role, insufficient or excessive autophagic activity can result in the development of diseases such as cancer and heart failure (Meijer and Codogno 2009; Jimenez et al. 2014; Bravo-San Pedro et al. 2017). In fact, autophagic vesicles are present in the heart tissue of patients with idiopathic dilated cardiomyopathy (Kostin et al. 2003) and defects in the autophagy-lysosomal pathway is a characteristic of Danon disease (Gustafsson and Gottlieb 2009). While autophagy can serve as an alternate pro-survival pathway to apoptosis, excessive autophagic flux can lead to apoptosis. Because of the complex nature of the process, it is not always clear whether autophagy is detrimental or beneficial in different situations, especially in stressful situations. However, autophagy is suggested to play a significant role in cardiac remodeling, particularly following MI. Studies have shown that autophagy promotes the survival of cardiac myocytes during myocardial ischemia and even reduces infarct size and attenuates adverse cardiac remodeling post-MI (Kanamori, Takemura, Goto, Maruyama, Ono, et al. 2011; Wu et al. 2014). Moreover, defects in autophagy have been linked to cardiac dysfunction and heart failure (Ilkun and Boudina 2013). Thus, autophagy is considered cardioprotective and a potential therapeutic target for the treatment of heart disease.

## Metabolism and Heart Failure

The heart is the most ATP-consuming organ in the body, utilizing 6 kg of ATP to pump 10 tons of blood daily (Wang et al. 2014; Lopaschuk 2016). Due to the low cardiac ATP reserve, the heart requires constant ATP synthesis to maintain contraction and relaxation (Wang et al. 2014). To achieve constant ATP synthesis, the heart utilizes a wide array of metabolic substrates like free fatty acids (FFAs), glucose, lactate, and even ketones (Nagoshi et al. 2011; Doenst et al. 2013; Wang et al. 2014; Lopaschuk 2016). Under normal physiological conditions, both fatty acids and carbohydrates like glucose serve as the main fuels to sustain cardiac function (Nagoshi et al. 2011; Lopaschuk 2016). However, under stressful conditions like ischemia, there is a shift in metabolic substrate utilization from FFA oxidation to carbohydrate oxidation, an effort that is thought to preserve the mechanical function and efficiency of the heart and enhance the recovery of post-ischemic function (Lopaschuk 2016). Interestingly this shift from FFA oxidation to carbohydrate oxidation, particularly glucose oxidation, is the normal adaptive response of the failing heart (Nagoshi et al. 2011; Doenst et al. 2013; Wang et al. 2014). However, studies have shown that there are no major metabolic substrate alterations in early-stage heart failure (Recchia et al. 1998; Funada et al. 2009; Wang et al. 2014). In fact, there are normal rates of FFA and glucose metabolism during the early stages of pacing-induced heart failure in dogs (Recchia et al. 1998). Furthermore, a study assessing metabolic substrate utilization by the failing human heart using arterio-venous blood sampling demonstrated that FFA uptake was similar in patients with early-stage heart failure compared to controls, a phenomenon that has mostly been attributed to the wide range of compensatory processes that occur during the early stages of heart failure (Funada et al. 2009). However, during end-stage or advanced decompensated heart failure FFA uptake is significantly reduced while glucose metabolism is increased (Wang et al. 2014), a

phenomenon that may be attributed to the fact that glucose metabolism is more oxygen efficient than FFA metabolism (Doenst et al. 2013). In fact, an evaluation of myocardial FFA and glucose uptake in patients with congestive heart failure revealed that FFA uptake decreased while glucose uptake increased in those heart (Taylor et al. 2001) and glucose metabolism is increased in cardiac hypertrophy (Kolwicz and Tian 2011). Thus, metabolic therapy has been proposed as a novel means of enhancing cardiac energetics and function in the failing heart (Nagoshi et al. 2011; Doenst et al. 2013; Wang et al. 2014). Because glucose uptake is increased during end-stage heart failure, therapies that promote glucose utilization while suppressing FFA metabolism have gained considerable attention (Nagoshi et al. 2011; Doenst et al. 2013; Wang et al. 2014).

## Ataxia-Telangiectasia Mutated Kinase and Ataxia Telangiectasia Disorder

Ataxia Telangiectasia Mutated Kinase (ATM) is a 370 kDa serine/threonine kinase located primarily in the nucleus where its primary function is to control cell cycle progression following double-stranded DNA breaks (Matsuoka et al. 1998; Rotman and Shiloh 1998; Yang and Kastan 2000; Abraham 2001; Peretz et al. 2001; Guinea Viniegra et al. 2005; Schneider et al. 2006; Halaby et al. 2008). Following DNA damage, ATM is activated via the autophosphorylation of several phosphorylation sites such as S2996, S367, S1893, and S1981 (Bakkenist and Kastan 2003; Kozlov et al. 2006). Active ATM then phosphorylates a plethora of downstream targets that are involved in cell cycle arrest (Banin et al. 1998; Matsuoka et al. 1998). For example, ATM activates p53 and Chk2, proteins critical in checkpoint-mediated cell cycle arrest (Banin et al. 1998; Matsuoka et al. 1998). Although the most widely known function of ATM is DNA damage repair, ATM also resides in the cytoplasm where it plays a critical role in regulating responses to many genotoxic stresses (Watters et al. 1999; Yang and Kastan 2000).

For instance, oxidative stress activates ATM (Guo et al. 2010). In fact, reactive oxygen species (ROS) can activate ATM directly (Guo et al. 2010). ROS levels are high in the cerebella of ATM-deficient mouse brains and there are abnormalities in antioxidant systems in ATM-deficient cells and tissues (Barzilai et al. 2002). Additionally, ATM-deficient mice exhibit alterations in levels of several compounds involved in oxidative stress (Barzilai et al. 2002). ATM-deficient mice experienced increased activity of thioredoxin coupled with a significant decrease in catalase activity and a significant increase in MnSOD (SOD2) in the cerebella (Barzilai et al. 2002). Such alterations indicate the progressive deterioration of the redox balance in ATM-deficient mice (Barzilai et al. 2002). ATM-deficient cells also exhibit mitochondrial dysfunction as they experience an increase in mitochondrial numbers, a phenomenon that has been attributed to impaired mitophagy (Valentin-vega et al. 2012).

Ataxia Telangiectasia (AT) is a rare multisystemic disease resulting from mutations in the *ATM* gene (Yang and Kastan 2000; Peretz et al. 2001; Halaby et al. 2008). Mutations in the *ATM* gene can result in either protein instability and complete functional loss or it can cause a decrease in the amount of functional protein or a decrease in kinase activity (Stewart et al. 2001; McKinnon 2004). The latter does not typically result in a severe phenotype as such mutations do not result in the complete absence of a functional ATM protein (Stewart et al. 2001; McKinnon 2004). Individuals with a mutation in one allele, ATM heterozygotes, make up roughly 1.4-2% of the population (Lavin et al. 1995; Khanna et al. 2001). Patients with AT can experience growth retardation, immunodeficiency, cancer susceptibility, cerebellar ataxia, insulin resistance, γ-radiation hypersensitivity, etc (Yang and Kastan 2000; Peretz et al. 2001; Guinea Viniegra et al. 2005; Schneider et al. 2006; Halaby et al. 2008). These individuals are also more susceptible to ischemic heart disease (Lavin et al. 1995; Khanna et al. 2001) and carriers of a mutated allele

are at a higher risk of death between ages 20 and 79 compared to non-carriers (Su and Swift 2000). On average, carriers of a mutates allele die 7 to 8 years younger than non-carriers with ischemic heart disease being the second cause of death in these individuals next to cancer (Su and Swift 2000).

## ATM and the Heart

### ATM expression in the heart

Catecholamines, such a norepinephrine, are released during myocardial ischemia and, when accumulated, may contribute to ischemic heart disease and heart failure (Foster, Singh, et al. 2012). Norepinephrine, acting via β-adrenergic receptor (β-AR) stimulation, induces cardiac myocyte apoptosis and myocardial remodeling (Singh et al. 2000; Krishnamurthy et al. 2007). In an effort to uncover the effect of β-AR stimulation on the expression of apoptosis-related genes, our laboratory used gene array technique to examine the expression of 96 apoptosis-related genes in sham and isoproterenol-infused hearts (isoproterenol is a synthetic catecholamine) (Foster, Singh, et al. 2012). It was found that β-AR stimulation decreased Bcl-2 expression but increased BNIP-3 expression in the heart (Foster, Singh, et al. 2012). However, involvement of BNIP-3 and Bcl-2 in cardiac myocyte apoptosis and myocardial remodeling had previously been examined (Gálvez et al. 2006; Diwan et al. 2007; Hamacher-Brady et al. 2007). What was interesting to observe was that ATM expression increased in hearts following β-AR stimulation with isoproterenol (Foster, Singh, et al. 2012). Furthermore, it was observed that ATM mRNA increased ~2.5 fold following β-AR stimulation with isoproterenol in cardiac myocytes (Foster, Singh, et al. 2012). Thus, our laboratory was the first lab to show that cardiac myocytes express ATM at basal levels and that β-AR stimulation increases ATM expression *in vivo* and *in vitro*

(Foster, Singh, et al. 2012). These data were further confirmed by increased expression of ATM in the heart following injuries, providing evidence that ATM protein levels increase in both the non-infarct and infarct regions of wild-type (WT) and ATM heterozygous knockout (hKO) hearts 1 and 3 days post-MI (Daniel et al. 2014).

ATM and heart function

Using ATM knockout (KO) mice to investigate the connection between the lack of ATM and basal cardiac structure and function, our laboratory previously provided evidence that ATM KO mice had lower left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular end-systolic volume (LVESV), systolic and diastolic septal wall thicknesses, and LV mass (Foster, Zha, et al. 2012). However, there were no significant changes in fractional shortening (%FS) and ejection fraction (%EF) (Foster, Zha, et al. 2012). Interestingly, myocardial fibrosis and myocyte cross-sectional area or hypertrophy, both key components in regulating heart function, were higher in KO hearts compared to WT hearts (Foster, Zha, et al. 2012). Since myocyte hypertrophy serves as a compensatory mechanism that improves heart function following hemodynamic overload (St et al. 2000), it is possible that %FS and EF are sustained in KO hearts by myocyte hypertrophy even though myocardial fibrosis is increased. These results also pointed in the direction of diastolic impairment in the absence of ATM under basal conditions.

Although the lack of ATM did not result in decreased in %FS and EF in the heart under basal conditions, studies investigating the role of ATM deficiency in cardiac function post-MI revealed that ATM deficiency does not have the same effect on cardiac remodeling early post-MI as it does late post-MI. In studies investigating the structure and function of the heart 1, 3, and 7 days post-MI in ATM hKO mice, m-mode echocardiography revealed that ATM hKO mice have

lower left ventricular (LV) diameters and volumes as well as increased %FS and EF 1, 3, and 7 days post-MI (Foster et al. 2013; Daniel et al. 2014). Additionally, ATM deficient mice experienced increased myocardial fibrosis and alpha-smooth muscle actin expression 3 and 7 days post-MI (Foster et al. 2013; Daniel et al. 2014). Alpha-smooth muscle action (α-SMA) is a marker of myofibroblast differentiation and serves as a key indicator of myocardial fibrosis as myofibroblasts are the primary source of fibrosis deposition post-MI (Foster et al. 2013; Daniel et al. 2014). Furthermore, ATM deficient hearts had increased infarct thickness 7 days post-MI (Foster et al. 2013), a phenomenon that may serve as a by-product of increased myofibroblast differentiation in ATM deficient hearts and aid in mitigating LV dysfunction early post-MI.

On the contrary, chronic MI resulted in LV dysfunction during ATM deficiency (Daniel et al. 2016). A study exploring cardiac function and remodeling in ATM hKO mice 14 and 28 days post-MI showed that ATM deficiency results in decreased %FS and EF as well as increased LVESV (Daniel et al. 2016). ATM deficient mice also had increased myocyte apoptosis, fibrosis, and myocyte hypertrophy (Daniel et al. 2016), all typical characteristics of heart failure during the later stages of MI. Interestingly, a similar phenomenon occurred in ATM deficient hearts 28 days following β-AR stimulation as evidenced by a decrease in %FS and EF together with increase in myocyte apoptosis and fibrosis (Foster, Singh, et al. 2012). Together, these results provide evidence that ATM is versatile, playing a protective role in the heart early after injury, but playing a detrimental role late after injury.

ATM and myocyte apoptosis and cardiac fibrosis

As the primary contractile cell in the heart, myocytes play a significant role in cardiac remodeling, putting myocyte apoptosis at the center of structural and functional changes in the heart during cardiac remodeling (Walker and Spinale 1999; Haunstetter and Izumo 2000; Nadal-

Ginard et al. 2003). p53 is a downstream target of ATM and activation of p53 prompts the expression of pro-apoptotic genes like Bax (Toshiyuki and Reed 1995). Investigation of the role of ATM deficiency in myocyte apoptosis 28 days post β-AR stimulation, ATM heterozygosity resulted in an increase in myocyte apoptosis (Foster, Singh, et al. 2012). However, the expression and phosphorylation of p53 and expression of Bax increased to a similar extent in WT and hKO hearts following β-AR stimulation (Foster, Singh, et al. 2012). Conversely, myocyte apoptosis increased roughly 0.4 fold in both WT and KO hearts 24 hours following β-AR stimulation (Foster, Zha, et al. 2012). In WT hearts only, β-AR stimulation resulted in the expression and phosphorylation of p53 and phosphorylation of JNK (Foster, Zha, et al. 2012). On the other hand, β-AR stimulation resulted in a decrease in Akt phosphorylation in KO hearts compared to WT hearts (Foster, Zha, et al. 2012). Together, these studies suggest that p53 and JNK pathways may be involved in β-AR-stimulated myocyte apoptosis in WT hearts, while Akt-dependent pathways may play a role in regulating apoptosis in KO hearts. Interestingly, Akt phosphorylation was lower in the infarct region of ATM deficient hearts 1 day post-MI compared to hKO-sham and WT-MI hearts (Daniel et al. 2014). This phenomenon further implicates Akt-dependent pathways in myocyte apoptosis during ATM deficiency, particularly post injury.

Myocardial fibrosis is characterized by an accumulation of extracellular matrix (ECM) proteins in the interstitium and plays an important role in the development of heart failure by acting on systolic and diastolic dysfunction (Kong et al. 2014). Fibroblasts produce enzymes such as matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) that influence ECM homeostasis and are key in the deposition of fibrosis in the myocardium (Fan et al. 2012). Myocardial fibrosis and MMP-2 protein levels significantly increased in ATM

deficient hearts under basal conditions and 28 days post-β-AR stimulation (Foster, Zha, et al. 2012; Foster, Singh, et al. 2012). However, TIMP-2, an MMP-2 inhibitor, decreased in ATM deficient hearts 28 days post-β-AR stimulation (Foster, Singh, et al. 2012). These results implicate MMP-2 in myocardial fibrosis at basal levels and following β-AR stimulation during ATM deficiency. Contrarily, ATM deficiency resulted in an increase in MMP-9 protein expression in the infarct region of the heart 7 days following MI (Foster et al. 2013). However, 28 days post-MI ATM deficiency resulted in an increase in fibrosis and a decrease in MMP-9 protein expression in the non-infarct region of the heart (Daniel et al. 2016). These results provide evidence that, unlike in the case of β-AR stimulation, MMP-9 plays a role in fibrosis post-MI.

ATM and cardiac inflammation

An inflammatory response is vital to cardiac remodeling and repair in response to insults such as MI (Frangogiannis 2008). Following MI, neutrophils infiltrate the infarcted area of the heart to remove dead cells (Frangogiannis 2008). Subsequently, macrophages engulf apoptotic neutrophils that lead to the production of anti-inflammatory cytokines (Frangogiannis 2008). One of the major anti-inflammatory cytokines produced is transforming growth factor β (TGF-β), which plays a role in myofibroblast differentiation and cardiac remodeling (Frangogiannis 2012).

Investigation of the role of ATM deficiency in the inflammatory response post-MI revealed that the number of neutrophils and macrophages dramatically increased in the infarct region of WT and hKO hearts 1 and 3 days post-MI compared to their respective shams (Daniel et al. 2014). Although there was no difference in the number of neutrophils and macrophages in the infarct region of hKO hearts 3 days post-MI, there was a significant decrease in the number of neutrophils and macrophages in the infarct region of hKO hearts 1 day post-MI (Daniel et al.

2014). Additionally, TGF-β protein expression was lower in the infarct region of hKO hearts 3 days post-MI (Daniel et al. 2014). Collectively, these studies provide evidence that deficiency of ATM delays the inflammatory response in the heart early following MI, which may help explain improved LV function early post-MI during ATM deficiency.

## ATM and Autophagy

As mentioned prior, autophagy is a catabolic process that functions to remove selected cytoplasmic components to maintain cellular homeostasis (Stagni et al. 2018). Autophagy is triggered by several phenomena, including nutrient starvation, mitochondrial dysfunction, hypoxia, etc (Stagni et al. 2018). Interestingly, cytoplasmic ATM is also activated in response to the same stressors (Stagni et al. 2018). In fact, ATM is shown to play a role in autophagy induction especially in cancer models (Farooqi et al. 2014; Stagni et al. 2018). Autophagy was induced in colorectal cancer cells treated with a low dose of camptothecin; furthermore, autophagy inhibitors enhanced apoptosis in these cell (Farooqi et al. 2014). Head and neck cancer cells treated with KU-55933, a specific ATM inhibitor, demonstrated increased autophagy and treating those cells with autophagy inducers increased KU-55933- induced apoptosis (Farooqi et al. 2014). Additionally, there is evidence that ATM activates the AMPK-ULK1 pathway, a pathway that is known to induce autophagy, in U87MG and U251 glioma cell lines (Farooqi et al. 2014). There is recent evidence suggesting that ATM mediates its effects on autophagy via mTOR regulation (Farooqi et al. 2014; Stagni et al. 2018). It has been demonstrated that ATM activates the TSC2 tumor suppressor via the LKB1/AMPK pathway in the breast cancer cell line MCF-7 to repress mTORC1 and induce autophagy in response to elevated ROS (Alexander et al. 2010). ATM is also shown to regulate autophagy by sustaining

levels and activity of ATG4C protease in cancer cells grown as mammospheres (Antonelli et al. 2017).

## ATM and Metabolism

AT patients have an increased risk of developing metabolic diseases such as hypertension, insulin resistance, impaired glucose metabolism, and diabetes (Stracker et al. 2013; Takagi et al. 2015; Dahl and Aird 2017). In fact, it has been shown that 17% of AT patients develop type 2 diabetes mellitus and $ATM^{-/-}$, $ATM^{+/-}$ /$ApoE^{-/-}$, and $ATM^{-/-}$ /$ApoE^{-/-}$ mice suffer from glucose intolerance (Takagi et al. 2015). In addition to showing that $ATM^{-/-}$ mice were glucose intolerant with a condition that mimicked type 2 diabetes mellitus, Takagi et al. also showed that $ATM^{+/-}$ male mice fed a high-fat diet had abnormal adipose distribution (Takagi et al. 2015). Analysis of brain glucose metabolism in humans with AT and their asymptomatic relatives revealed fluctuations in glucose metabolism in AT patients compared to their asymptomatic relatives and unrelated controls (Volkow et al. 2014). AT patients had lower metabolism in the fusiform gyrus, cerebellar hemispheres, hippocampus, and anterior vermis compared to siblings or controls (Volkow et al. 2014). AT patients had higher metabolism in the globus pallidus that associated with negative motor performance compared to controls or siblings (Volkow et al. 2014).

ATM signaling has been linked to inulin signaling and subsequent metabolic regulation (Bar et al. 1978; Dahl and Aird 2017). Bar et al. showed that monocytes of AT patients have a decreased binding affinity for insulin when compared to their unaffected counterparts (Bar et al. 1978). It has been proposed that ATM signaling through p53 plays a critical role in both insulin resistance and glucose homeostasis (Dahl and Aird 2017). Once activated, ATM phosphorylates and activates p53 which in turn can suppress glycolysis via a plethora of pathways (Dahl and

Aird 2017). p53 can regulate the *SLC2A* and *SLC2A4* genes that encode for the glucose transporters GLUT1 and GLUT4 (Dahl and Aird 2017). p53 can also inhibit IKK and activate TIGAR to regulate glycolysis (Dahl and Aird 2017). Additionally, KU-55933, a specific ATM inhibitor, increased glucose uptake and lactate production in MCF-7 and HepG2 cells (Zakikhani et al. 2012). KU-55933 also decreased levels of TCA metabolites fumarate, malate, citrate, and α-ketoglutarate in MCF-7 cells, while increasing succinate levels in those cells (Zakikhani et al. 2012). Collectively, these results place ATM as a critical player in cellular energy metabolism.

## Specific Aims

The overall goal of this study was to determine the role of ATM in cardiac autophagy and glucose metabolism under ischemic conditions. The specific aims of this study were to: (1) investigate the role of ATM deficiency in autophagy early (4 hours) during MI; (2) examine the role of ATM deficiency in autophagy late (28 days) post-MI; and (3) determine the role of ATM deficiency in glucose metabolism post-ischemia.

CHAPTER 2

# ATAXIA-TELANGIECTASIA MUTATED KINASE DEFICIENCY IMPAIRS AUTOPHAGIC RESPONSE EARLY DURING MYOCARDIAL INFARCTION

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Abstract

Ataxia-telangiectasia mutated kinase (ATM) is activated in response to DNA damage. We have previously shown that ATM plays a critical role in myocyte apoptosis and cardiac remodeling following myocardial infarction (MI). Here, we tested the hypothesis that ATM deficiency results in autophagic impairment in the heart early during MI. MI was induced in wild-type (WT) and ATM heterozygous knockout (hKO) mice by ligation of the left anterior descending artery (LAD). Structural and biochemical parameters of the heart were measured 4 hours following LAD ligation. M-mode echocardiography revealed that MI worsens heart function as evidenced by reduced percent ejection fraction and fractional shortening in both groups. However, MI-induced increase in left ventricular end-diastolic and systolic diameters, and volumes were significantly lower in hKO hearts. ATM deficiency resulted in autophagic impairment during MI as evidenced by decreased LC3-II, increased p62, decreased cathepsin D protein levels, and increased aggresome accumulation. ERK1/2 activation was only observed in WT-MI hearts. Activation of Akt and AMPK was lower, whereas activation of GSK-3β and mTOR was higher hKO-MI hearts. Inhibition of ATM using KU-55933 resulted in autophagy impairment in cardiac fibroblasts as evidenced by decreased LC3-II protein levels and formation of acidic vesicular organelles. This impairment associated with decreased activation of Akt and AMPK, but enhanced activation of GSK-3β and mTOR in KU-treated fibroblasts. Thus, ATM deficiency results in autophagic impairment in the heart during MI and cardiac fibroblasts. This autophagic impairment may occur via the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK.

## New & Noteworthy

ATM plays a critical role in myocyte apoptosis and cardiac remodeling following myocardial infarction (MI). Here, we provide evidence that ATM deficiency results in autophagic impairment during MI. Further investigation of the role of ATM in autophagy post-MI may provide novel therapeutic targets for Ataxia-telangiectasia patients suffering from heart disease.

## Introduction

Heart failure is the leading cause of death worldwide. Cardiovascular disease accounted for ~17.3 million deaths out of 54 million total deaths worldwide in 2013. Despite having medications to manage the complications of heart failure, there is currently no treatment to alleviate the condition (18). Coronary artery disease (CAD) is the most common form of heart disease with myocardial infarction (MI) as a serious outcome. Treatment of CAD and its progression to heart failure remains a challenging area of research (17).

Ataxia-telangiectasia mutated kinase (ATM) is a ~370 kDa serine/threonine kinase that primarily resides in the nucleus (19, 20, 40, 44, 45, 54). The main function of ATM is to control cell cycle progression following DNA damage, particularly double strand breaks (1, 36). In response to DNA damage, ATM is activated and recruited to DNA double strand breaks. Although ATM has mostly been reported as a nuclear protein involved in signaling pathways that control DNA damage recognition, it is also located in the cytoplasm where it plays a role in several metabolic pathways (50, 54). Mutations in ATM cause a multisystemic disease known as Ataxia-telangiectasia (AT) (20, 40, 54). AT heterozygotes, individuals with an ATM mutation in one allele, make up a substantial portion of the population (~1.4 to 2%) (25, 30). The incidence of AT is significantly higher in consanguineous populations (6). Although AT heterozygotes are spared from most of the symptoms of AT, they are more susceptible to ischemic heart disease (25, 30).

Macroautophagy (hereafter called autophagy) is a conserved physiological process in the body that ultimately results in the packaging of cytoplasmic components into autophagosomes that fuse to lysosomes for degradation (5, 23, 29, 37). Autophagy consist of three main phases: 1)

induction and phagophore formation, 2) phagophore elongation and autophagosome formation, and 3) lysosomal fusion, degradation, and recycling (38). Autophagy is critical to cellular homeostasis under normal and stressful conditions, and plays a crucial role in the development of diseases such as cancer and heart failure (5, 23, 37). In fact, autophagy is suggested to play a significant role in cardiac remodeling during MI. Studies have shown that autophagy promotes the survival of cardiac myocytes, reduces infarct size, and attenuates adverse cardiac remodeling post-MI (24, 52). Moreover, defects in autophagy have been linked to cardiac dysfunction and heart failure (22). Thus, autophagy is considered cardioprotective and a potential therapeutic target for the treatment of heart disease.

Autophagic changes occur in mouse heart as early as 30 minutes following ligation of the left anterior descending artery (LAD). The LC3II-to-LC3-I ratio, an established indicator of autophagic turnover, is maximum 4 hours following LAD ligation (24), suggesting that autophagic activity increases in the ischemic heart during the early stages of MI. Previously we have shown that ATM plays a critical role in myocyte apoptosis and cardiac remodeling following β-adrenergic receptor stimulation and MI (10, 11, 14–16). Here, we tested the hypothesis that deficiency of ATM impairs autophagic response in the heart early during MI (4 hours after its onset). The data presented here suggest that ATM deficiency impairs autophagy in the heart during MI via the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK.

## Materials and Methods

### Vertebrate Animals

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the experiments were performed in accordance with the protocols approved by the East Tennessee State University Committee on Animal Care. ATM deficient mice (129xblack Swiss hybrid background), generated as described (4), were purchased from Jackson Laboratory. Genotyping was performed by PCR using primers suggested by the Jackson Laboratory. Age-matched (~ 4-month-old) male and female mice were used for the study. Since homozygous knockout mice die at approximately 2 months of age due to thymic lymphomas (4), the study used ATM heterozygous knockout (hKO; deficient) mice. All mice undergoing surgery (Sham and MI) received buprenorphine injections prior to surgery.

### Myocardial Infarction

Myocardial infarction (MI) was performed as previously described (10). Briefly, mice were anesthetized with a mixture of isoflurane (2%) and oxygen (0.5 l/min) inhalation and ventilated using a rodent ventilator (Harvard Apparatus). Body temperature was maintained at ~37°C using a heating pad. The heart was exposed by a left thoracotomy. The left anterior descending coronary artery (LAD) was ligated using a 7-0 polypropylene suture. Sham-operated mice underwent the same procedure without ligation of the LAD. At the end of the study period (4 hours following LAD ligation), isolated hearts were used for either histology or molecular analyses.

Echocardiography

M-mode echocardiography images were obtained using transthoracic short-axis view imaging at midpapillary level 4 hours during MI using a Vevo 1100 machine utilizing a 550D probe. M-mode tracings were used to calculate percent fractional shortening (%FS) and ejection fractions (%EF) and measure heart rate, LV wall thicknesses, end-systolic (LVESD) and end-diastolic dimensions (LVEDD), and end-systolic (LVESV) and end-diastolic volumes (LVEDV). An individual blinded to the experimental groups recorded the images, while a second individual assessed the images and calculated the structural and functional parameters of the heart.

Aggresome detection by histological staining

Paraffin-embedded heart sections were deparaffinized and rehydrated prior to staining. The tissue sections were immersed in xylene to remove the paraffin. Subsequently, the sections were rehydrated with decreasing ethanol concentrations (100, 90, 80%). The sections were stained using Proteostat dye (2000x dilution in PBS from the Proteostat Aggresomes Detection Kit, Enzo) for 3 minutes. The tissue sections were then washed in deionized water and destained using 1% acetic acid for 20 minutes. After washing with deionized water and PBS, slides were mounted using anti-fade mounting media, and visualized using the EVOS FL Auto fluorescence microscope (ThermoFisher Scientific). The images were acquired at 20x magnification. The number of aggresomes were quantified in the entire LV area.

Western blot analysis

LV or cell lysates were prepared in RIPA buffer [10 mM Tris·HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride] supplemented with protease inhibitors. Equal amounts of proteins (25 μg) were resolved using SDS-PAGE. The proteins were then transferred to a PVDF membrane. The membrane was blocked for one hour using 5% nonfat milk and incubated overnight with primary antibodies against LC3B (1:1000), p62 (1:1000), p-Akt (ser-473; 1:1000), p-GSK-3β (ser-9; 1:1000), p-mTOR (ser-2448; 1:1000), p-ERK1/2 (1:1000) (Cell Signaling), cathepsin D (1:1000), or p-AMPK (thr-172; 1:1000) (Santa Cruz). The immune-complexes were detected using appropriate secondary antibodies and chemiluminescent reagents. Protein signals were visualized using ImageQuant LAS 500 imager and quantified using ImageQuant TL software (GE). Equal protein loading for phosphoproteins was verified by reprobing/probing the membranes using anti-Akt, anti-GSK-3β, anti-AMPK, anti-mTOR, anti-ERK1/2 and anti-GAPDH antibodies. Quantitative analyses showed no differences in total protein levels for these proteins among the groups. Therefore, all the western blot data is normalized using GAPDH as a loading control.

Fibroblast isolation and treatment

Adult rat cardiac fibroblasts were isolated as previously described (53). The cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and serum starved for 5 hours before use. The cells were then treated with the ATM inhibitor KU-55933 (KU; 100 μM; Tocris) for 4 hours. The cells were maintained in serum-free DMEM for the duration of the treatment.

Evaluation of acidic vesicular organelles by acridine orange

At the end of the study period, fibroblasts treated with KU for 4 hours were incubated with 1 μg/mL acridine orange (Sigma) in serum-free DMEM for 15 minutes. The cells were then washed three times using PBS and observed using the EVOS FL Auto fluorescence microscope (ThermoFisher Scientific). All images were acquired at 20x magnification. Occurrence of acidic vesicular organelles was measured by the intensity of orange fluorescence using NIS-Elements image analysis software (Nikon). Aggresomes were quantified by counting cells in 15 randomly chosen fields per dish for each experiment.

Statistical analysis

Data are expressed as means ± SE. Data were analyzed using Student's t-test or a two-way analysis of variance followed by the Student-Newman-Keuls test. P values of $<0.05$ were considered to be significant.

## Results

Echocardiographic measurements

M-mode echocardiography showed no difference in echocardiographic parameters between the two sham groups. MI significantly decreased %EF (WT-sham, 59.26±2.39; hKO-sham, 61.09±2.43; WT-MI, 31.59±5.75*; hKO-MI, 26.95±5.16*; *$p<0.05$ vs. respective sham; n=5) and FS (WT-sham, 30.96±1.66; hKO-sham, 32.17±1.69; WT-MI, 14.95±2.97*; hKO-MI, 12.29±2.69*; *$p<0.05$ vs. respective sham; n=5) in both groups compared to their respective shams. However, there was no significant difference in %EF and FS between the two genotypes post-MI (Figure 2.1A-2.1B). MI increased LVEDD (WT-sham, 3.19±0.17; hKO-sham,

3.36±0.33; WT-MI, 3.62±0.10*; hKO-MI, 3.07±0.16$^{\#}$; *p<0.05 vs respective sham; $^{\#}$p<0.05 vs WT-MI; n=5), LVESD (WT-sham, 2.21±0.13; hKO-sham, 2.30±0.26; WT-MI, 3.09±0.18*; hKO-MI, 2.65±0.14$^{\#}$; *p<0.05 vs respective sham; $^{\#}$p<0.05 vs WT-MI; n=5), LVEDV (WT-sham, 41.70±4.76; hKO-sham, 49.10±10.67; WT-MI, 55.67±3.47*; hKO-MI, 38.41±5.02$^{\#}$; *p<0.05 vs respective sham; $^{\#}$p<0.05 vs WT-MI; n=5), and LVESV (WT-sham, 17.06±2.30; hKO-sham, 19.90±4.85; WT-MI, 36.68±5.41*; hKO-MI, 26.44±3.42$^{\#}$; *p<0.05 vs respective sham; $^{\#}$p<0.05 vs WT-MI; n=5) in WT when compared to WT-sham group. However, there was no significant increase in LVEDD, LVESD, LVEDV, and LVESV in hKO-MI versus hKO-sham group. In fact, these parameters were significantly lower in the hKO-MI versus WT-MI group (Figure 2.1C-2.1F). Heart rates, and posterior and septal wall thicknesses were not found to be significantly different between the two MI groups (data not shown).

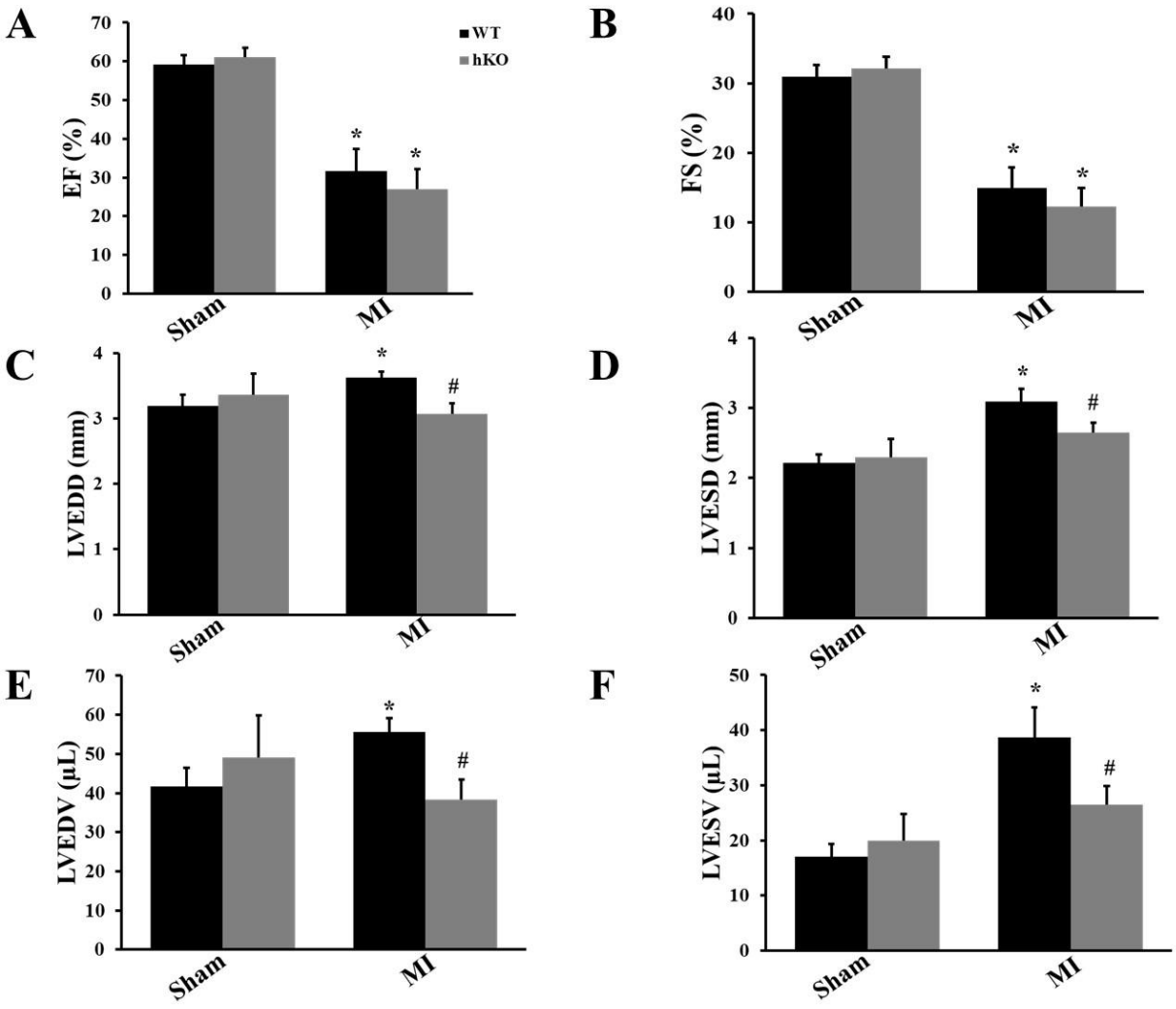


Figure 2.1. Echocardiographic analysis of heart function. MI was performed in WT and hKO mice. Indices of cardiac function; percent ejection fraction (%EF), percent fractional shortening (%FS), LVEDD, LVESD, LVEDV, and LVESV were calculated using echocardiographic images 4 hours following LAD ligation. *A:* %EF. *B:* %FS. *C:* LVEDD. *D:* LVESD. *E:* LVEDV. *F:* LVESV. *p<0.05 vs respective sham; #p<0.05 vs WT-MI; *n*=5. LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume.

Expression of autophagy-related proteins in cardiac tissue

LC3 is considered as one of the most important autophagy markers since it is essential to autophagosome formation (39). In mammalian cells, LC3 is processed into LC3-I, a soluble form of LC3. LC3-I is then modified to LC3-II, a membrane-bound form of LC3, by the addition of

phosphatidylethanolamine (PE). To date, LC3-II is the only well-characterized protein that is specifically localized to autophagosomes throughout the autophagy process, from autophagosome formation to lysosomal degradation. Thus, measuring the levels of LC3-II protein is suggested to be a good marker for early autophagosome formation (39). Western blot analysis of LV lysates using an anti-LC3 antibody revealed a significant increase in LC3-II protein levels in hKO-sham versus the WT-sham group (Figure 2.2). LC3-II protein levels were significantly increased in the WT-MI group when compared to the WT-sham. In contrast, LC3-II protein levels were significantly lower in the hKO-MI group when compared to the hKO-sham and WT-MI groups (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; n=4-7; Figure 2.2).

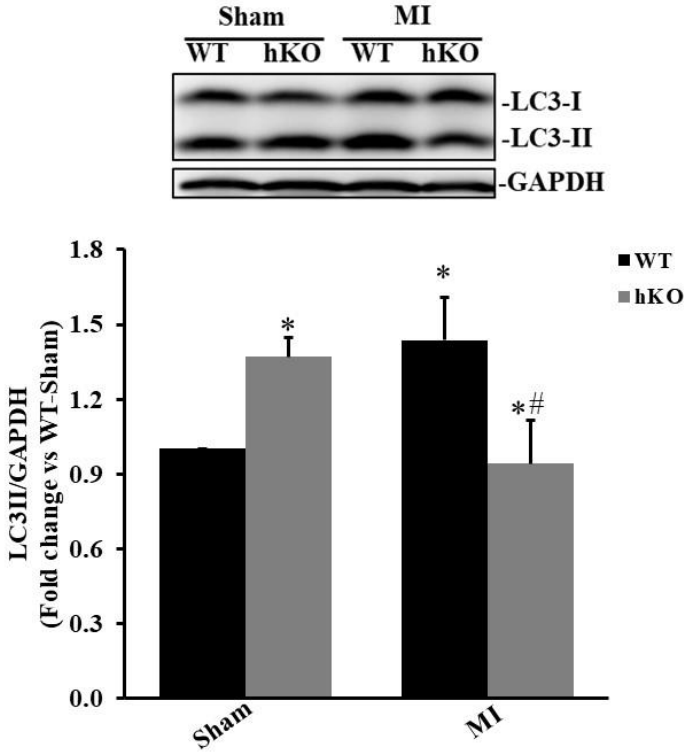


Figure 2.2. ATM deficiency reduces LC3-II protein levels 4 hours during MI. Total LV lysates, prepared from sham and MI hearts, were analyzed by western blot using LC3B antibodies. *Top*: western blot exhibiting immunostaining for LC3-I, LC3-II and GAPDH. *Bottom*: quantitative analysis of LC3-II normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; *n=4-7*).

p62 is an autophagy receptor, meaning that it recognizes and transports misfolded proteins to the autophagosome for degradation by lysosomes (33). Cathepsin D, a lysosomal protease, is involved in lysosomal degradation of misfolded proteins (49). Both p62 and cathepsin D are critical to autophagic clearance or the degradation of autophagolysosomes. p62 protein levels were significantly higher in the hKO-sham group when compared to its WT counterpart (Figure 2.3A). p62 protein levels remained unchanged in WT-MI group. However, p62 protein levels were significantly greater in hKO-MI group versus the hKO-sham and WT-MI groups (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; n=5-7; Figure 2.3A). Cathepsin D protein levels were not significantly different between the two sham groups. Cathepsin D protein levels remained unchanged in WT-MI group. However, MI led to a significant decrease in cathepsin D levels in hKO group versus hKO-sham and WT-MI groups (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; n=4-5; Figure 2.3B).

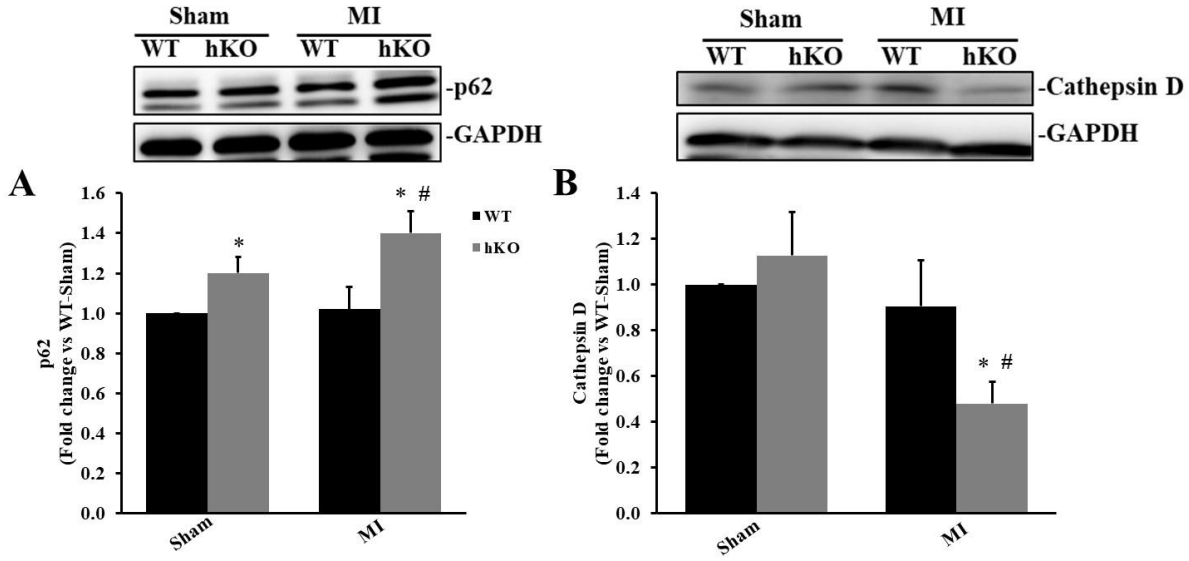


Figure 2.3. ATM deficiency increases p62 protein levels and decreases cathepsin D protein levels 4 hours during MI. Total LV lysates, prepared from sham and MI hearts, were analyzed by western blot using anti-p62 (A) and anti-cathepsin D (B) antibodies. Both p62 bands were analyzed. *Top:* western blots exhibiting immunostaining for p62, cathepsin D, and GAPDH. *Bottom:* quantitative analyses of p62 and cathepsin D protein levels normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; *n=4-7*).

Activation of signaling molecules related to autophagy

Typically, Akt signaling acts as a positive regulator of autophagy (47). However, there are several downstream targets of Akt, such as GSK-3β, AMPK, and mTOR, that can act to either upregulate or downregulate the autophagy pathway. Inactivation (phosphorylation) of GSK-3β induces autophagy (7, 35, 51), while activation (phosphorylation) of AMPK stimulates autophagy (27) and activation (phosphorylation) of mTOR inhibits autophagy (27, 28). Phosphorylation of Akt remained unchanged in the sham groups and in the WT group post-MI. However, phosphorylation of Akt was significantly lower in the hKO-MI group when compared to hKO-sham and WT-MI groups (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-MI; n=5-7; Figure 2.4A). Akt acts as an upstream regulator of GSK-3β, phosphorylating it at serine 9 and inactivating it (13). Phosphorylation of GSK-3β was significantly lower in the hKO-MI group when compared to hKO-sham and WT-MI groups (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-MI; n=5-7; Figure 2.4B). AMPK phosphorylation was significantly lower in the hKO-MI group when compared to hKO-sham and WT-MI groups (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-MI; n=5-7; Figure 2.4C). mTOR phosphorylation was significantly lower in hKO-sham vs WT-sham. MI significantly increased mTOR phosphorylation in both genotypes versus their respective sham groups. However, phosphorylation of mTOR was significantly greater in hKO-MI when compared to WT-MI group (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-MI; n=4-5; Figure 2.4D).

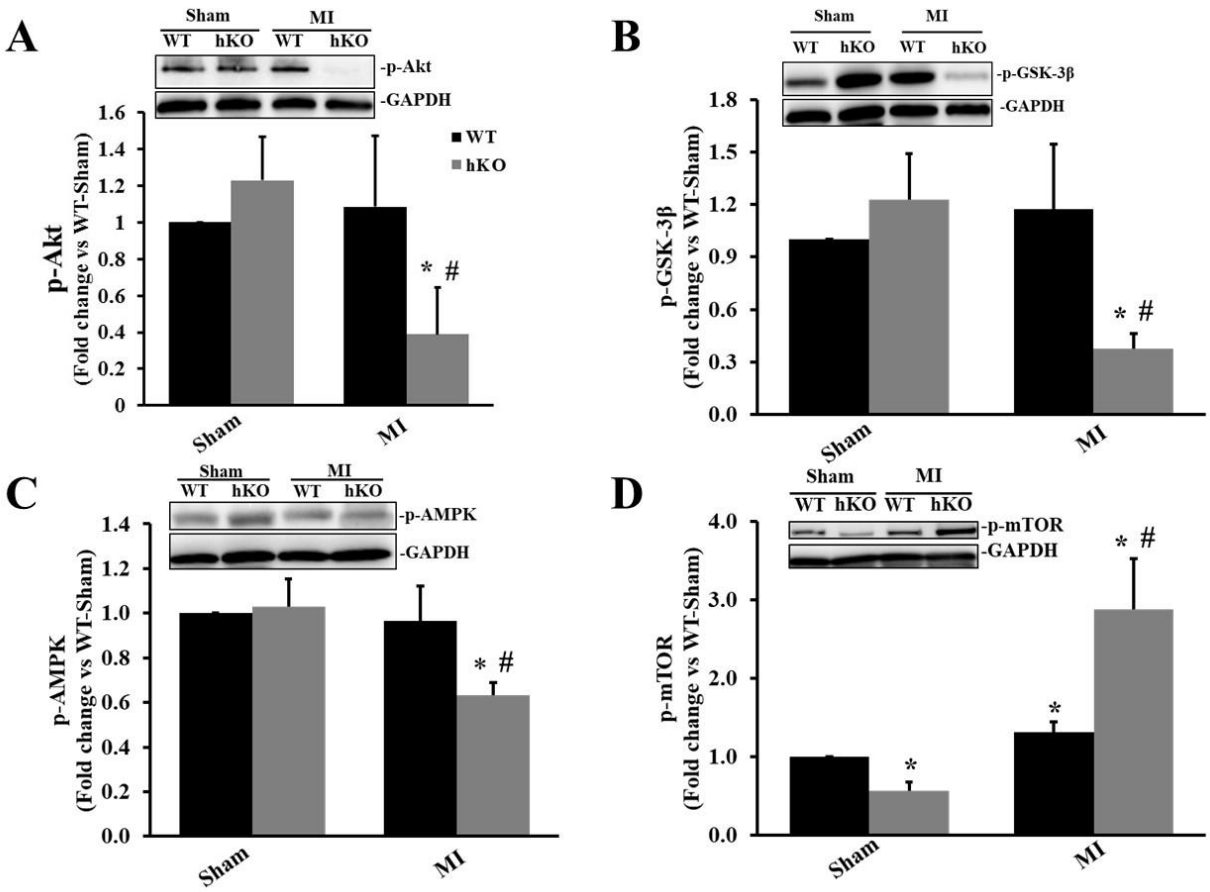


Figure 2.4. ATM deficiency affects activation of Akt, GSK-3β, AMPK, and mTOR 4 hours during MI. Total LV lysates, prepared from sham and MI hearts, were analyzed by western blot using anti-p-Akt (A), anti-p-GSK-3β (B), anti-p-AMPK (C), and anti-p-mTOR (D) antibodies. *Top:* western blots exhibiting immunostaining for p-Akt, p-GSK-3β, p-AMPK, p-mTOR, and GAPDH. *Bottom:* quantitative analyses of p-Akt, p-GSK-3β, p-AMPK, and p-mTOR normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; *n=4-7*).

ERK1/2 activation acts as a positive regulator of autophagy (47). ERK1/2 phosphorylation was not significantly different between the two sham groups. ERK1/2 phosphorylation was significantly higher in the WT-MI compared to the WT-sham group (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; n=7; Figure 2.5). It remained unchanged between the hKO-MI vs hKO-sham group.

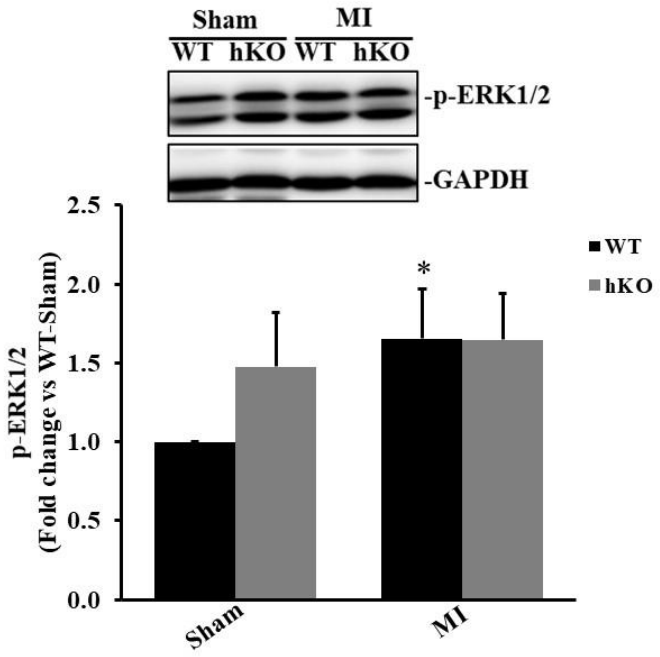


Figure 2.5. ERK1/2 activation 4 hours during MI. Total LV lysates, prepared from sham and MI hearts, were analyzed by western blot using phospho-specific ERK1/2 antibodies. *Top*: western blot exhibiting immunostaining for p-ERK1/2 and GAPDH. *Bottom*: quantitative analysis of p-ERK1/2 normalized to GAPDH (*p<0.05 vs respective sham; *n=7*).

Aggresome formation in cardiac tissue

Aggresomes are aggregates of misfolded proteins within cells, which can ultimately be degraded by the autophagy pathway (46). The number of aggresomes was significantly lower in hKO-sham when compared to WT-sham group. MI led to decreased number of aggresomes in WT-MI versus WT-sham group. However, the number of aggresomes was significantly higher in hKO-MI group when compared to hKO-sham and WT-MI groups (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; n=3; Figure 2.6).

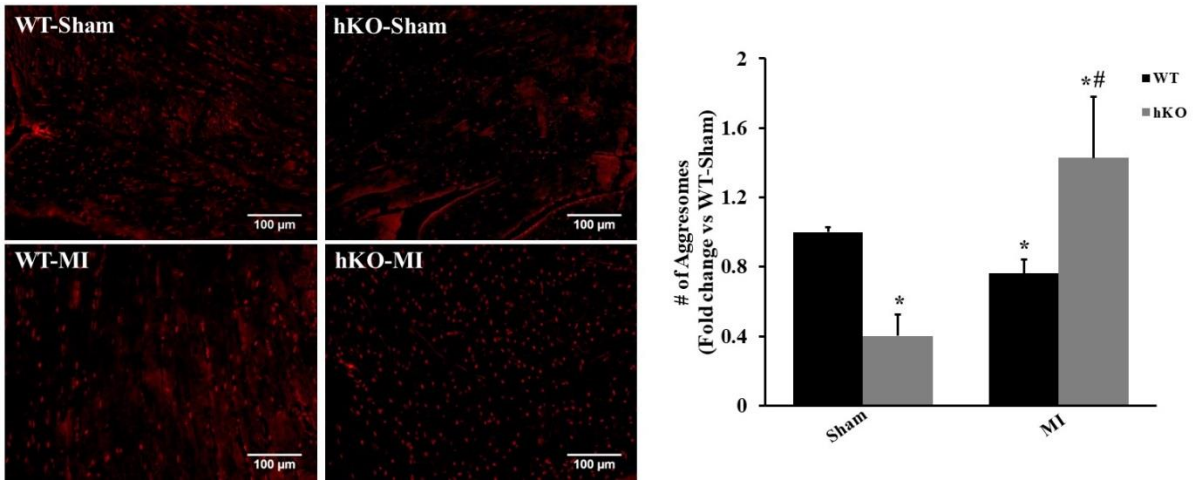


Figure 2.6. ATM increases aggresome accumulation in the LV 4 hours during MI. *Left:* Proteostat dye-stained images obtained from the LV of WT and hKO hearts 4 hours during MI. Red fluorescent staining indicates aggresomes. *Right:* quantitative analysis of aggresomes in the LV region (*p<0.05 vs respective sham; #p<0.05 vs WT-MI; *n=3*).

Autophagic response in cardiac fibroblasts

Cardiac fibroblast-specific deletion of ATM in mice is shown to play a pivotal role in doxorubicin-induced cardiotoxicity (55). In fact, cardiac fibroblasts serve as a major cell type involved in deposition of fibrosis in the heart post-MI (48). To investigate if inhibition of ATM affects autophagic response of cardiac fibroblasts, serum-starved cultures of cardiac fibroblasts were treated with KU (100 μM) for 4 hours. Western blot analyses of cell lysates showed a significant decrease in LC3-II protein levels in KU-treated cells (*p<0.05 vs CTL; n=3; Figure 2.7). ATM inhibition had no effect on p62 and cathepsin D protein levels (data not shown).

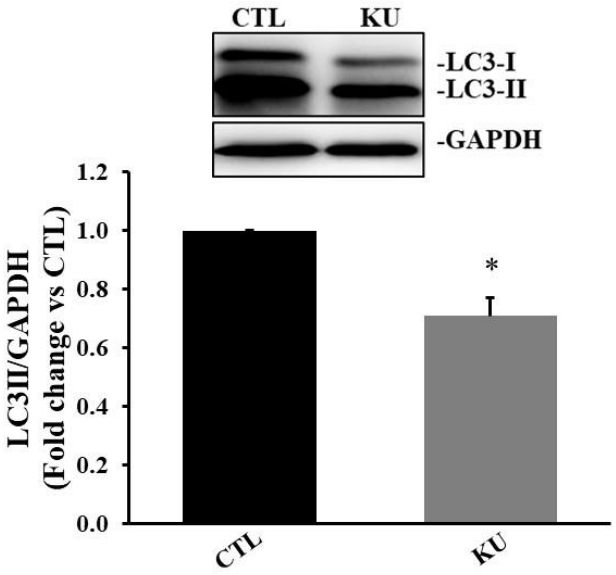


Figure 2.7. Inhibition of ATM reduces LC3-II protein levels in cardiac fibroblasts. Serum-starved cardiac fibroblasts were treated with KU-55933 (100 μm; ATM inhibitor) for 4 hours. Total cell lysates were analyzed by western blot using LC3B antibodies. *Top*: western blot exhibiting immunostaining for LC3-I, LC3-II and GAPDH. *Bottom*: quantitative analysis of LC3-II normalized to GAPDH (*$p<0.05$ vs CTL; n=3).

KU treatment significantly decreased phosphorylation of Akt, GSK-3β, and AMPK (*$p<0.05$ vs CTL; n=3; Figure 2.8A-2.8C). However, it significantly increased mTOR phosphorylation (*$p<0.05$ vs CTL; n=3; Figure 2.8D).

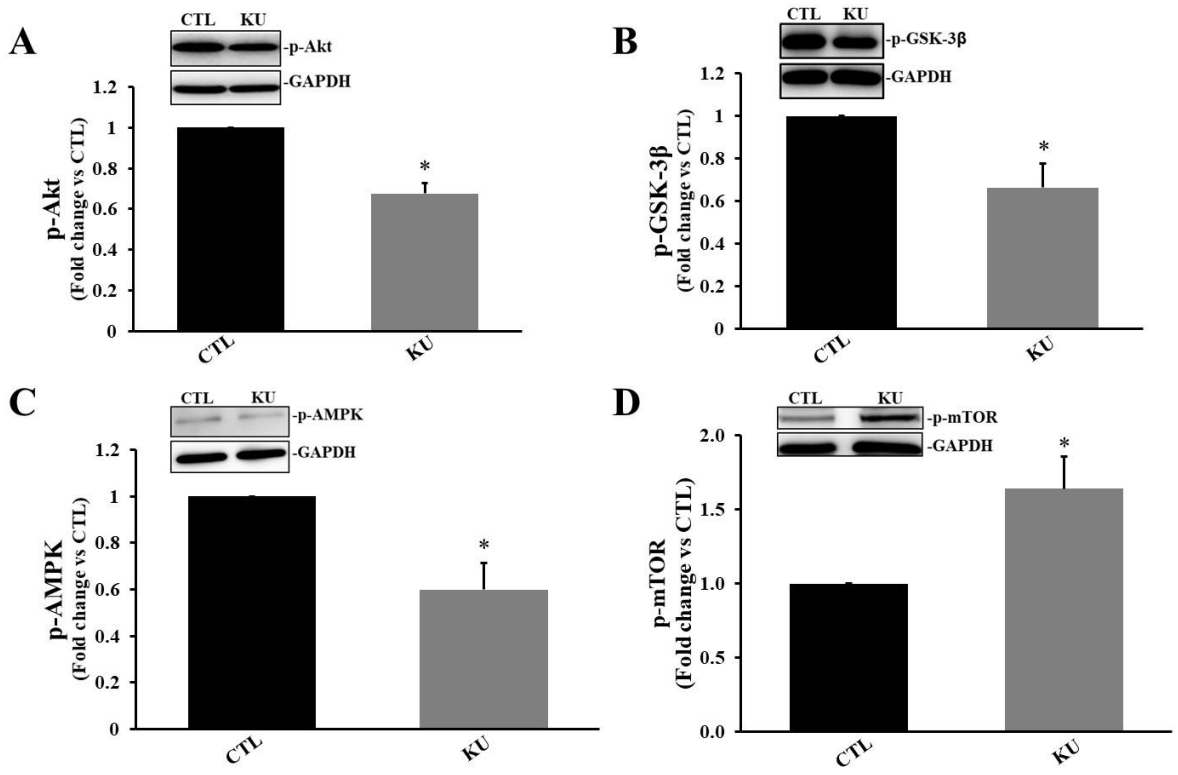


Figure 2.8. Inhibition of ATM affects activation of Akt, GSK-3β, AMPK, and mTOR in cardiac fibroblasts. Serum-starved cardiac fibroblasts were treated with KU-55933 (100 μm; ATM inhibitor) for 4 hours. Total cell lysates were analyzed by western blot using anti-p-Akt (A), anti-p-GSK-3β (B), anti-p-AMPK (C), and anti-p-mTOR (D) antibodies. *Top:* western blots exhibiting immunostaining for p-Akt, p-GSK-3β, p-AMPK, p-mTOR, and GAPDH. *Bottom:* quantitative analyses of p-Akt, p-GSK-3β, p-AMPK, and p-mTOR normalized to GAPDH (*$p<0.05$ vs CTL; n=3).

The formation of acidic vesicular organelles (AVOs) is a key characteristic of cells that have passed through autophagy. AVOs, such as autolysosomes, increase upon autophagy induction (43). KU significantly decreased AVO formation in cardiac fibroblasts 4 hours post-treatment (*$p<0.05$ vs CTL; n=3; Figure 2.9).

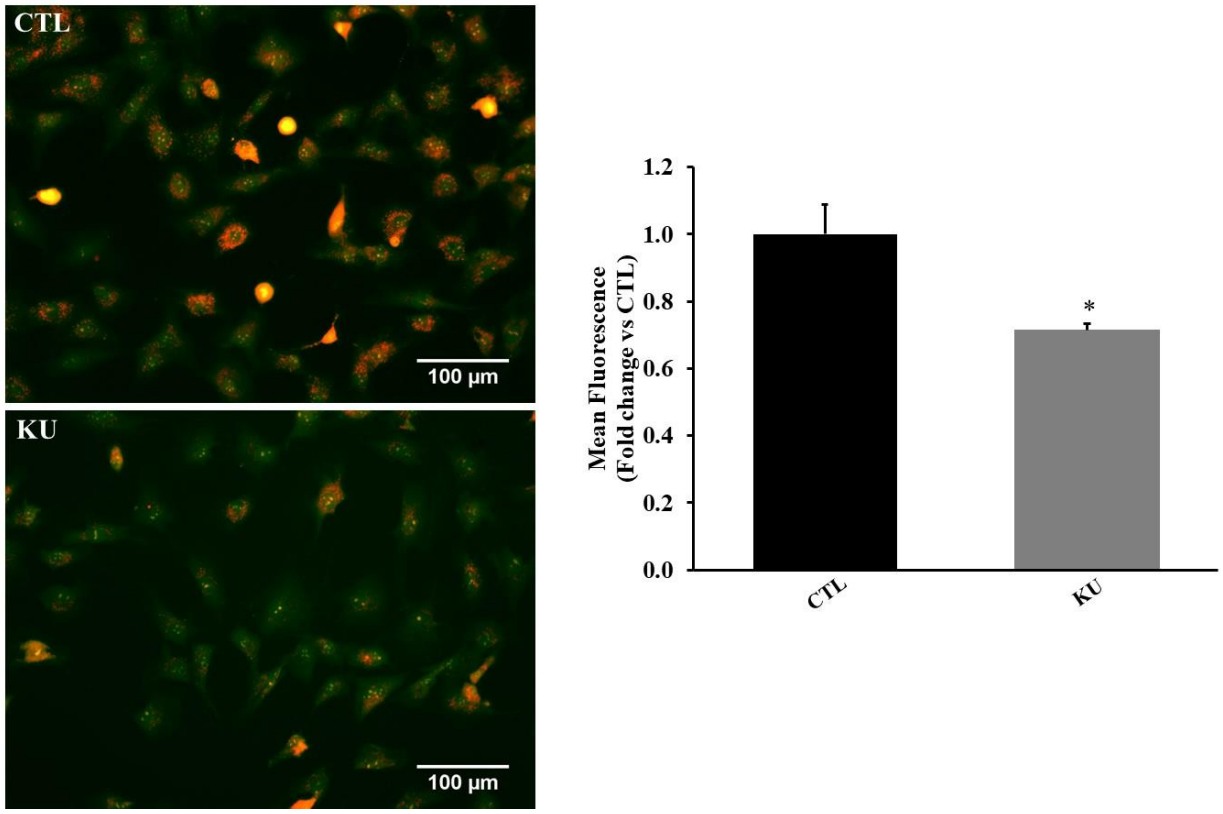


Figure 2.9. Inhibition of ATM decreases acidic vesicular organelle (AVOs) formation in cardiac fibroblasts. Cardiac fibroblasts were treated with KU-55933 (100 µm) for 4 hours and then stained with acridine orange dye. *Left*: Orange fluorescent staining indicates acidic vesicular organelles (AVOs). *Right:* quantitative analysis of acidic vesicular organelles (AVOs) in cardiac fibroblasts (*$p<0.05$ vs CTL; n=3).

## Discussion

ATM typically becomes activated in the presence of double-stranded DNA breaks (1, 36). Thus, most of the studies regarding ATM and autophagy have been in relation to the DNA damage response. This is the first study investigating the role of ATM in cardiac autophagy, particularly during MI. A major finding of this study is that ATM deficiency results in autophagic impairment 4 hours during MI. This is evidenced by decreased LC3-II, increased p62, decreased cathepsin D protein levels, and enhanced aggresome accumulation. In accordance with *in vivo* data, ATM inhibition also resulted in autophagic impairment in adult cardiac

fibroblasts as shown by a reduction in LC3-II protein expression and decrease in acidic vesicular organelle (AVO) formation. Further, we provide evidence that ATM deficiency may exert its effect on autophagy during MI via the activation of GSK-3β and mTOR and inactivation of Akt and AMPK as phosphorylation of Akt, GSK-3β, and AMPK decreased during MI, while mTOR phosphorylation increased during MI.

MI worsened heart function 4 hours during MI as evidence by a decrease in %EF and FS in both groups when compared to their respective sham group. This is consistent with the observations of de Andrade et al. (2015) in CD1 mice where MI led to decreased %EF and FS 4 hours following LAD ligation (3). Previously we provided evidence that ATM deficiency improves left ventricular (LV) function 1 day following LAD ligation as observed by hKO-MI hearts exhibiting higher %EF and FS coupled with lower LVESV and LVEDV when compared to WT-MI group (10). Here, %EF and FS remained unchanged between the two MI groups 4 hours following LAD ligation. The observed no significant difference in %EF and FS between WT and hKO hearts may relate to an early observation time point (4 hours vs 24 hours after LAD ligation).

Autophagy is suggested to be cardioprotective, reducing infarct size and attenuating adverse cardiac remodeling post-MI (24, 52). Moreover, defects in autophagy have been linked to cardiac dysfunction and heart failure (22). Thus, autophagy is typically upregulated post-MI (2, 24, 52). Specifically autophagy is induced in the acute phase of MI but impaired in the later phase of MI (24, 52). However, the mechanism(s) by which these autophagic changes occur post-MI is unknown. In our study, MI resulted in autophagic induction early during MI in WT hearts as evidenced by high LC3-II protein levels coupled with reduced aggregate accumulation.

Interestingly, Akt, GSK-3β, and AMPK phosphorylation was unaffected in WT hearts during MI. However, phosphorylation of mTOR (activation) and activation of ERK1/2 were significantly higher in WT hearts during MI. Several studies suggest that ERK1/2 signaling positively regulates autophagy (21, 47, 57). However, the involvement of mTOR in ERK1/2-mediated autophagic changes are not completely clear. Zhang et al. showed that mTOR inhibition results in ERK1/2 activation that induces autophagy in hepatocellular carcinoma (56). Choi et al. demonstrated that ERK1/2 activation induced autophagy in luteal cells independent of mTOR activation (9). Additionally, ERK1/2 activation results in autophagic impairment in irinotecan-induced steatohepatitis (34). In the current study, we provide evidence that ERK1/2 activation may be involved in the induction of autophagy in WT hearts during MI. However, further investigations are needed to define the correlation between ERK1/2 and mTOR activation.

ATM is suggested to play a role in autophagy, particularly following DNA damage in cancer models. ATM is shown to be necessary for autophagy induction in response to ionizing radiation in Hela cells, a human cervical cancer cell line (32). ATM-mediated phosphorylation of PTEN promoted autophagy in cancer cells in response to DNA-damage causing agents (8). ATM/AMPK signaling promoted autophagy induction in mouse spermatocytes in response to cadmium-induced oxidative stress (31). In the present study, we provide evidence that ATM is critical in cardiac autophagy at basal levels and during MI. ATM deficiency induces autophagy at basal levels as indicated by increased LC3-II protein levels coupled with an accumulation of p62. However, the number of aggresomes were lower in hKO-sham versus WT-sham group. Thus, it is possible that under basal conditions ATM deficiency results in enhanced autophagic flux; such enhancement could result in the accumulation of LC3-II and p62 and the rapid

degradation of aggresomes. During MI, ATM deficiency resulted in reduced LC3-II, increased p62, decreased cathepsin D protein levels and enhanced aggresome accumulation. Similarly, ATM inhibition resulted in a reduction in the LC3-II protein levels in cardiac fibroblasts. Although inhibition of ATM had no effect on p62 and cathepsin D protein expression in cardiac fibroblasts, the formation of acidic vesicular organelles was reduced. Together, these results suggest that ATM deficiency impairs autophagy in the heart at all three stages (autophagy induction, autophagosome formation, and autophagolysosome degradation) during MI and in cardiac fibroblasts. In ATM deficient hearts during MI, initiation of autophagy is lower as evidenced by decreased LC3-II. The enhanced accumulation of p62 and aggresomes is likely due to decreased cathepsin D levels, indicating impairment in aggresome degradation. In KU-treated cardiac fibroblasts, initiation of autophagy is decreased as evidenced by decreased LC3-II levels. The decrease in acidic vesicular organelles coupled with no changes in p62 and cathepsin D suggests a lack of autophagosome formation and subsequent degradation. Thus ATM deficiency impairs autophagy in the heart during MI and in cardiac fibroblasts by inhibiting autophagy induction, autophagosome formation, and autophagolysosome degradation. These findings ultimately reveal that ATM is critical in cardiac autophagy regulation in the presence and absence of an insult.

The Akt/GSK-3β pathway is well known as a pro-survival pathway as it typically prevents apoptotic signaling (12). Akt signaling has also been implicated in the regulation of autophagy. Akt activation induces autophagy in the brain following middle cerebral artery occlusion, resulting in neuroprotection (42). Pharmacological inhibition of GSK-3β induces pro-survival autophagic signals in human pancreatic cancer cells (35). Additionally, knockdown of GSK-3β increases basal autophagy and AMPK signaling in human aortic endothelial cells (51).

AMPK functions as an energy sensor in cells that is activated by ATP depletion or glucose starvation (41). In the heart, AMPK activation is critical to the heart's response to stresses such as ischemia (41). Pharmacological activation of AMPK has cardioprotective effects, protecting the heart against ischemia-reperfusion injury by maintaining ATP levels post ischemia and reducing infarct size (26). AMPK activation promotes autophagy (27). Under conditions of low energy levels and hypoxia, AMPK inhibits mTOR (the master regulator of autophagy) activity via the phosphorylation of Raptor (7, 28). In the present study, we provide evidence that autophagic enhancement during ATM deficiency under basal conditions does not involve alterations in Akt, GSK-3β, or AMPK phosphorylation as those parameters remained unchanged in ATM deficient hearts. However, mTOR phosphorylation was lower in ATM deficient hearts, indicating that enhanced autophagic response in ATM deficient hearts under basal conditions may be mTOR dependent. ATM deficiency may cause autophagic impairment during MI and in cardiac fibroblasts via the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK. We suggest that inactivation of Akt causes a reduction in GSK-3β phosphorylation, thus activating GSK-3β. Increased GSK-3β activity may result in a decline in AMPK phosphorylation and activation. This decline in AMPK activation results in mTOR activation that ultimately inhibits autophagic response. However, further investigations are necessary to confirm the relationship between the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK in regulating autophagy during MI in ATM deficient hearts.

In summary, WT hearts exhibited autophagy induction during MI, a phenomenon most likely because ERK1/2 activation. Under basal conditions, ATM deficiency results in enhanced autophagic response that appears to be mTOR dependent. In addition, ATM deficiency results autophagic impairment in all phases (autophagy induction, autophagosome formation, and

autophagolysosome degradation) during MI and in cardiac fibroblasts via the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK. It should be emphasized that our data on ATM deficiency and cardiac autophagy are obtained 4 hours following LAD ligation. The experimental time point should be extended beyond 4 hours to investigate the long-term effects of ATM deficiency on autophagic changes in the heart.


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## Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

## Author Contributions

P.R.T., M.S., and K.S. conception and design of research; P.R.T., S.L.S., C.C.C., and S.D. performed experiments; P.R.T., S.L.S., and C.C.C. analyzed data; P.R.T. and K.S. interpreted results of experiments; P.R.T. prepared figures; P.R.T. drafted manuscript; P.R.T., M.S., and

K.S. edited and revised manuscript; P.R.T., S.L.S., S.D., C.C.C., M.S., and K.S. approved final version of manuscript.

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CHAPTER 3

# ATAXIA-TELANGIECTASIA MUTATED KINASE DEFICIENCY AUGMENTS AUTOPHAGIC RESPONSE IN THE INFARCT REGION IN A CHRONIC MYOCARDIAL INFARCTION MODEL

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## Abstract

Ataxia-telangiectasia mutated kinase (ATM) is a cell cycle checkpoint protein activated in response to oxidative stress and DNA damage. We have previously shown that ATM deficiency impairs autophagic response in the heart early during myocardial infarction (MI). Here, we tested the hypothesis that ATM deficiency augments autophagy in the heart in a chronic MI (28 days post-MI) setting. MI was induced in wild-type (WT) and ATM heterozygous knockout (hKO) mice by ligating the left anterior descending artery (LAD). Levels of LC3-II and p62, major markers of autophagy, remained unchanged in the non-infarct region of the heart. Interestingly, MI altered autophagy in the infarct region of both WT and hKO hearts as evidenced by increased LC3-II and Beclin protein levels coupled with decreased p62 protein levels. However, Levels of LC3-II and cathepsin D were significantly higher in hKO-infarct hearts versus WT-infarct. Activation of AMPK was higher in the WT-Infarct group, while activation of Akt and mTOR was higher in the hKO-infarct versus WT-Infarct group. In vitro, treatment of cardiac fibroblasts using KU-55933, an inhibitor of ATM, augmented autophagy as evidenced by increased LC3-II protein levels and decreased p62 protein levels. Thus, ATM deficiency results in autophagic augmentation in the infarct region of the heart 28 days post-MI and in cardiac fibroblasts.

## Introduction

Heart failure is the number one cause of death worldwide (12). Myocardial infarction (MI) is the most common cause of heart failure (31). Following MI, the heart undergoes remodeling, a process that ultimately changes the architecture and composition of the left ventricle(33). Macroautophagy, hereafter referred to as autophagy, is a degradative process in the body that serves to maintain cellular homeostasis by packaging cytoplasmic components into autophagosomes for eventual degradation by lysosomes (6, 16, 20, 26). Autophagy maintains homeostasis under normal conditions and is suggested to play a critical role in the development of pathologies such as cancer, diabetes, and heart failure (6, 15, 26). It is shown to play a key role in myocyte apoptosis and cardiac remodeling post-MI (16, 43). Autophagy is activated in cardiac myocytes within 30 minutes following permanent coronary ligation in a mouse model (16). Autophagic activity was particularly higher in myocytes bordering the infarcted region (16). Furthermore, pharmacological inhibition of autophagy increased infarct size by 31% (16), while pharmacological activation of autophagy attenuated cardiac remodeling and dysfunction 7 and 21 days post-MI (43). Thus, autophagy is considered cardioprotective and could serve as a therapeutic strategy for both acute and chronic MI.

Ataxia-telangiectasia mutated kinase (ATM) is a serine/threonine kinase typically activated in response to DNA damage (1, 25). Although it is primarily located in the nucleus, it is suggested to have cytoplasmic functions including regulating responses to an array of genotoxic stresses in addition to mediating several metabolic pathways (4, 13, 39, 46). Individuals with a mutation in the *ATM* gene develop a multisystemic disease known as Ataxia Telangiectasia (AT) (14, 29, 46). These individuals make up roughly 1.4 to 2% of the population (17, 22) and are at a

higher risk of death between ages 20 and 79 (34). Previously, we examined the role of ATM in autophagy early (4 hours) during MI (38). It was observed that ATM deficiency impairs autophagic response under basal conditions as well as following MI (38). This impairment in autophagy during ATM deficiency associated with activation of GSK-3β and mTOR, and inactivation of Akt and AMPK (38). In the present study, we tested the hypothesis that ATM deficiency augments autophagic response in the heart late post-MI (28 days after its onset). The data presented here suggests that ATM deficiency augments autophagy in the infarcted region of the heart 28 days post-MI, which associates with the activation of Akt and mTOR.

## Materials and Methods

### Vertebrate Animals

This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experiments were performed in accordance with the protocols approved by the East Tennessee State University Committee on Animal Care. ATM deficient mice (129xblack Swiss hybrid background), generated as described (3), were purchased from Jackson Laboratory. Age-matched (aged ~4 months) female and male mice were used for the study. Since homozygous knockout mice die at roughly 2 months of age from thymic lymphomas (3), the study used ATM heterozygous knockout (hKO; deficient) mice. All mice undergoing surgery (Sham and MI) received buprenorphine injections prior to and 24 hours following surgery.

### Myocardial Infarction

Myocardial infarction (MI) was performed as previously described (9). Briefly, mice were anesthetized with a mixture of isoflurane (2%) and oxygen (0.5 l/min) inhalation and ventilated using a rodent ventilator (Harvard Apparatus). Body temperature was maintained at ~37°C using a heating pad. The heart was exposed by a left thoracotomy. The left anterior descending coronary artery (LAD) was ligated using a 7-0 polypropylene suture. Sham-operated mice underwent the same procedure without ligation of the LAD. At the end of the study period (28 days following LAD ligation), isolated hearts were separated into infarct and non-infarcted LV regions for molecular analyses.

Fibroblast isolation and treatment

Adult rat cardiac fibroblasts were isolated as previously described (45). The cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. The cells were serum starved for 5 hours prior to use. The cells were then treated with the ATM inhibitor KU-55933 (KU; 100 μM; Tocris) for 24 hours. The cells were maintained in serum-free DMEM for the duration of the treatment.

Western blot analysis

LV or cell lysates were prepared in RIPA buffer [10 mM Tris·HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, and 0.2 mM phenylmethylsulfonyl fluoride] supplemented with protease inhibitors. Equal amounts of proteins (25 μg) were resolved using SDS-PAGE. The proteins were then transferred to a PVDF membrane. The membrane was blocked for one hour using 5% nonfat milk and incubated overnight with primary antibodies against LC3B (1:1000), p62 (1:1000), p-Akt (ser-473; 1:1000), p-GSK-3β (ser-9; 1:1000), p-mTOR (ser-2448; 1:1000), p-Erk1/2 (1:1000) (Cell Signaling), cathepsin D (1:1000), Beclin (1:000), or p-AMPK (thr-172; 1:1000) (Santa Cruz). The immune-complexes were detected using appropriate secondary antibodies and chemiluminescent reagents. There were no significant differences in GAPDH levels between genotypes or groups, therefore GAPDH (1:10,000; Cell Signaling) immunostaining was used as the protein loading control. Protein signals were visualized using ImageQuant LAS 500 imager and quantified using ImageQuant TL software (GE).

## Statistical analysis

Data are expressed as means ± SE. Data were analyzed using Student's t-test or a two-way analysis of variance followed by the Student-Newman-Keuls test. P values of $<0.05$ were considered to be significant.

# Results

## Expression of autophagy-related proteins in cardiac tissue

LC3 is considered to be one of the most important markers of autophagy as it is essential to autophagosome formation (28). In mammalian cells, LC3 is processed to LC3-I and subsequently LC3-II by the addition of phosphatidylethanolamine (PE) (28). p62, an autophagy receptor, aids in the tagging and transportation of misfolded proteins to the autophagosome by working in conjunction with the ubiquitin-proteasome pathway (23). Western blot analysis showed increased levels of LC3-II in hKO-sham versus WT sham (Fig 3.1A). However, LC3-II protein levels remained unchanged in the non-infarct LV in both genotypes when compared to the respective sham groups. No significant differences were observed in p62 protein levels among the four groups (Fig 3.1B). In the infarct LV region, there was a significant increase in LC3-II protein levels in both MI groups. Interestingly, the levels of LC3-II protein were significantly greater in hKO-MI group versus WT-MI (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-Inf; n=4-5; Fig 3.2A). The levels p62 were decreased to a similar extent in the two MI groups when compared to their respective shams (*$p<0.05$ vs respective sham; n=4-5; Fig 3.2B). Based on these observations, infarct LV lysates were used for further molecular analysis.

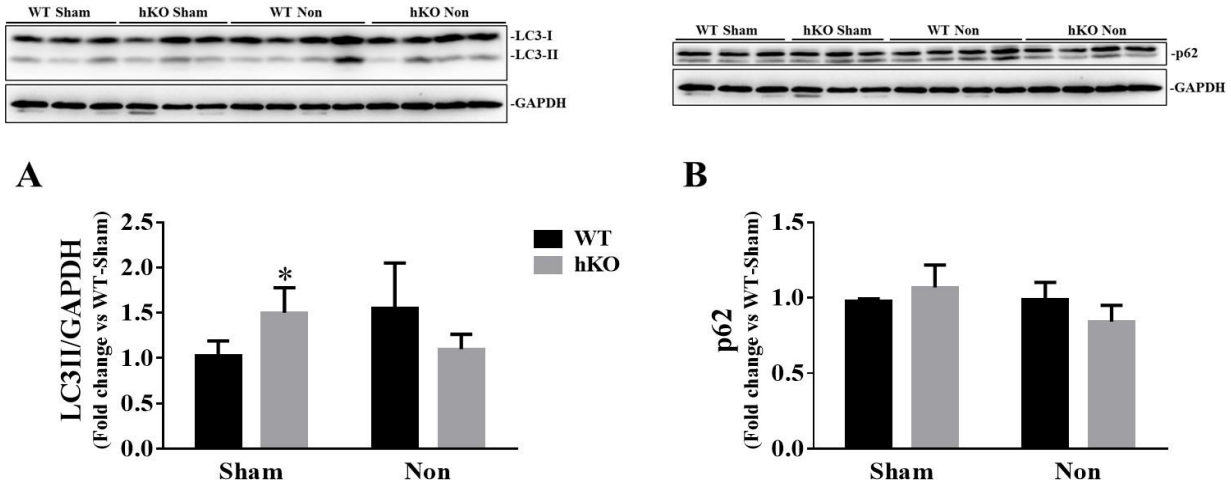


Figure 3.1. LC3-II and p62 protein levels 28 days post-MI in the non-infarct region. Total LV lysates, prepared from sham and infarct (Non) regions post-MI, were analyzed by western blot using anti-LC3B (A) and anti-p62 (B) antibodies. *Top:* western blots exhibiting immunostaining for LC3-I, LC3-II, p62, and GAPDH. *Bottom:* quantitative analyses of LC3-II and p62 protein levels normalized to GAPDH (*p<0.05 vs respective sham; *n=3-4*).

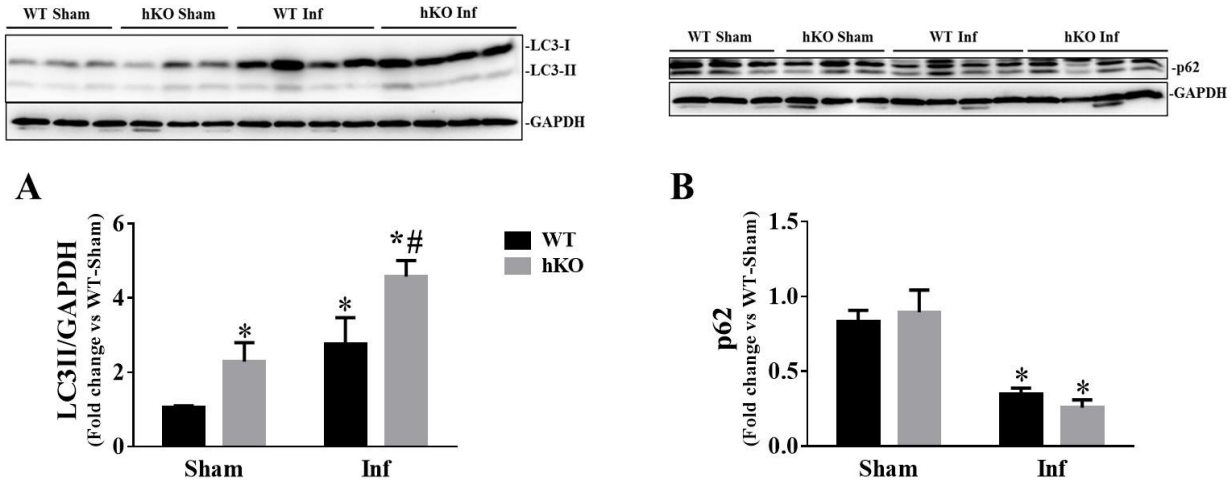


Figure 3.2. LC3-II and p62 protein levels 28 days post-MI in the infarct region. Total LV lysates, prepared from sham and infarct (Inf) regions post-MI, were analyzed by western blot using anti-LC3B (A) and anti-p62 (B) antibodies. *Top:* western blots exhibiting immunostaining for LC3-I, LC3-II, p62, and GAPDH. *Bottom:* quantitative analyses of LC3-II and p62 protein levels normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-Inf; *n=4-5*).

Beclin, a mammalian ortholog of the yeast autophagy-related gene 6 (Atg6), also plays a critical role in autophagosome formation (42). Beclin acts during the initiation stage of

autophagy by forming the isolation membrane, a double-membrane structure that engulfs cytoplasmic material to form the autophagosome (42). Although there was no significant difference in Beclin protein levels in hKO-sham versus WT-sham group, Beclin protein levels were increased to a similar extent in the infarct region of both WT and hKO groups (*p<0.05 vs respective sham; n=3-4; Fig 3.3A). Cathepsin D is a lysosomal endopeptidase involved in lysosomal degradation of misfolded proteins (37). p62 and cathepsin D play a critical role in autophagic clearance or autophagolysosome degradation. Cathepsin D protein levels were also not significantly different between the two sham groups (Fig 3.3B). Cathepsin D protein levels remained unchanged in the infarct region of WT-MI group, however, there was a significant increase in cathepsin D protein levels in hKO-MI group versus WT-MI group (#p<0.05 vs WT-Inf; n=3-4; Fig 3.3B).

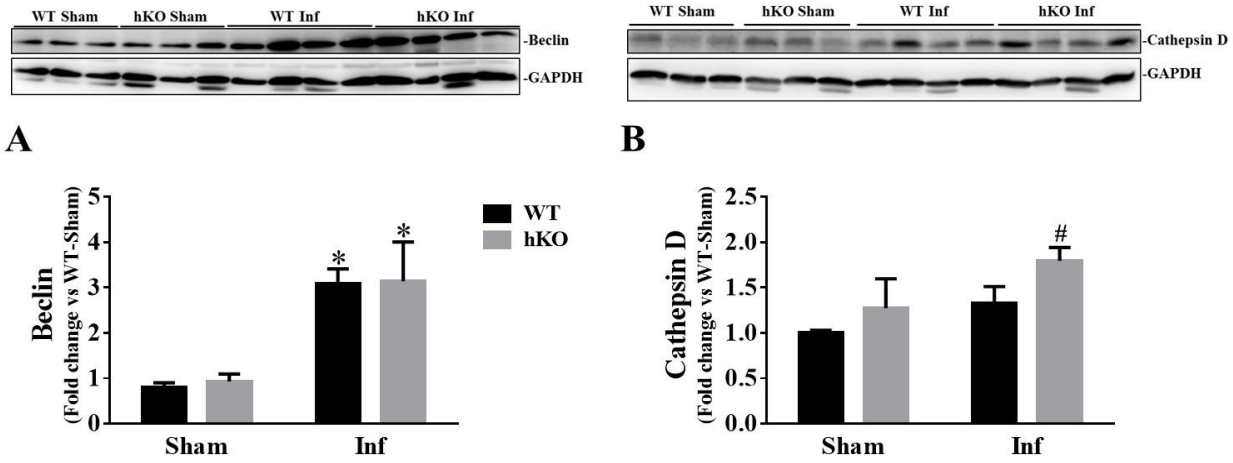


Figure 3.3. Beclin and cathepsin D protein levels 28 days post-MI in the infarct region. Total LV lysates, prepared from sham and infarct (Inf) regions post-MI, were analyzed by western blot using anti-Beclin (A) and anti-cathepsin D (B) antibodies. *Top:* western blots exhibiting immunostaining for Beclin, cathepsin D, and GAPDH. *Bottom:* quantitative analyses of Beclin and cathepsin D protein levels normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-Inf; *n=3-4*).

Activation of signaling molecules related to autophagy

Typically, Akt, AMPK, and Erk1/2 signaling are all positive regulators of autophagy (7, 24, 35, 41), while mTOR is a well-known negative regulator of autophagy (18, 19). Phosphorylation of Akt, AMPK, Erk1/2, and mTOR was not significantly different between the two sham groups (*$p<0.05$ vs respective sham; #$p<0.05$ vs WT-Inf; n=3-4; Fig 3.4A-D). However, phosphorylation of Akt was greater in the infarct region of hKO-MI, not in WT-MI, group versus hKO-sham (Fig 3.4A). On the other hand, AMPK phosphorylation was significantly greater in in the infarct region of WT-MI, not in hKO-MI, group versus WT-sham and hKO-MI (Fig 3.4B). MI led to a similar increase in Erk1/2 phosphorylation in the infarct region of both genotypes (Fig 3.4C). Increased phosphorylation of mTOR was only observed in the infarct region of hKO-MI group which was found to be significantly greater versus the WT-MI group (Fig 3.4D).

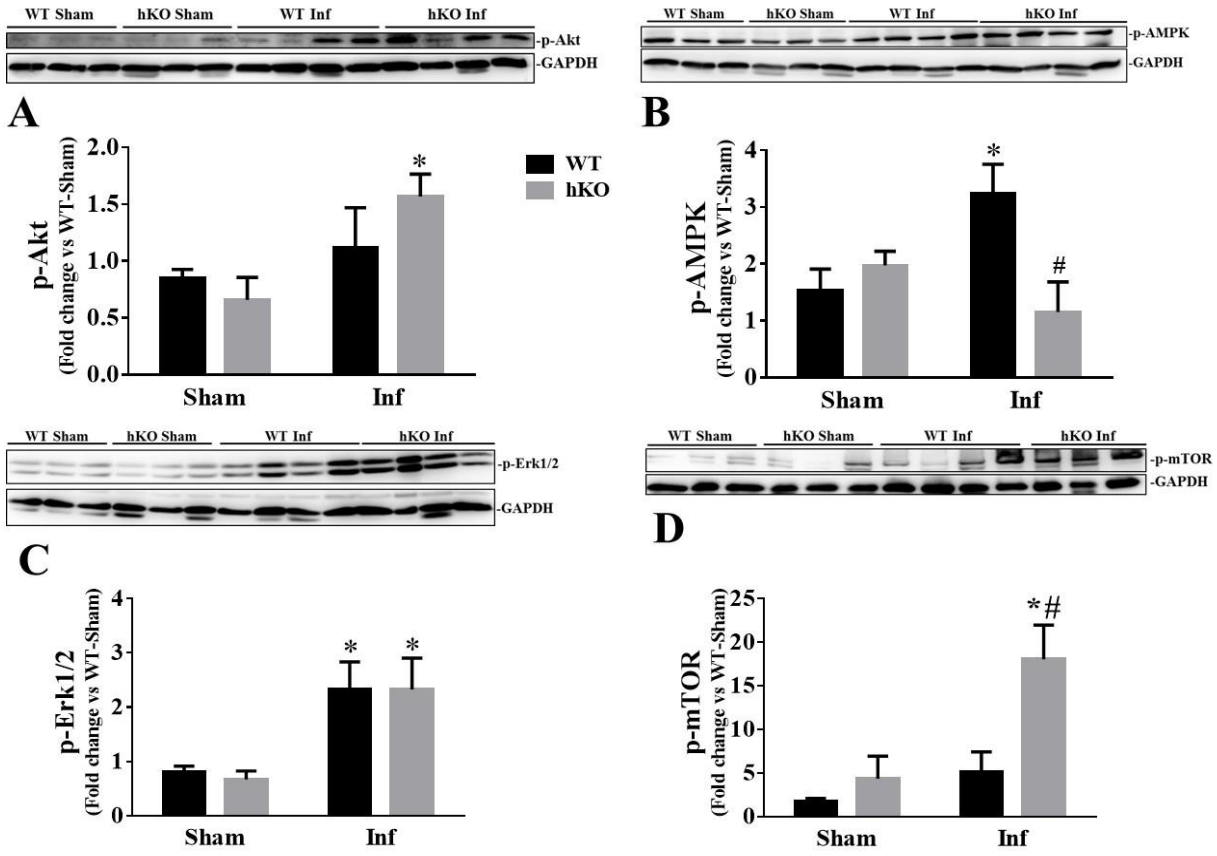


Figure 3.4. Activation of Akt, AMPK, Erk1/2, and mTOR 28 days post-MI in the infarct region. Total LV lysates, prepared from sham and infarct (Inf) regions post-MI, were analyzed by western blot using anti-p-Akt (A), anti-p-AMPK (B), anti-p-Erk1/2 (C), and anti-p-mTOR (D) antibodies. *Top:* western blots exhibiting immunostaining for p-Akt, p-AMPK, p-Erk1/2, p-mTOR, and GAPDH. *Bottom:* quantitative analyses of p-Akt, p-AMPK, p-Erk1/2, and p-mTOR normalized to GAPDH (*p<0.05 vs respective sham; #p<0.05 vs WT-Inf; *n=3-4*).

Autophagic response in cardiac fibroblasts

Cardiac fibroblasts play an important role in fibrosis deposition in the heart post-MI (36). Fibroblasts participate in the healing process of the heart by differentiating into myofibroblasts (5). Myofibroblasts are found in the infarct region of the heart following MI and aid in wound closure (8). Previously, we found that inhibition of ATM with KU (100 μM) for 4 hours negatively regulated autophagy in cardiac fibroblasts (38). Here, we investigated if autophagy is positively or negatively regulated in cardiac fibroblasts 24 hours following treatment with ATM

inhibitor, KU-55933 (KU). Western blot analyses of cell lysates showed a significant increase in LC3-II (*p<0.05 vs CTL; n=4; Figure 3.5A) protein levels coupled with a significant decrease in p62 protein levels (*p<0.05 vs CTL; n=4; Figure 3.5B) in KU-treated cells.

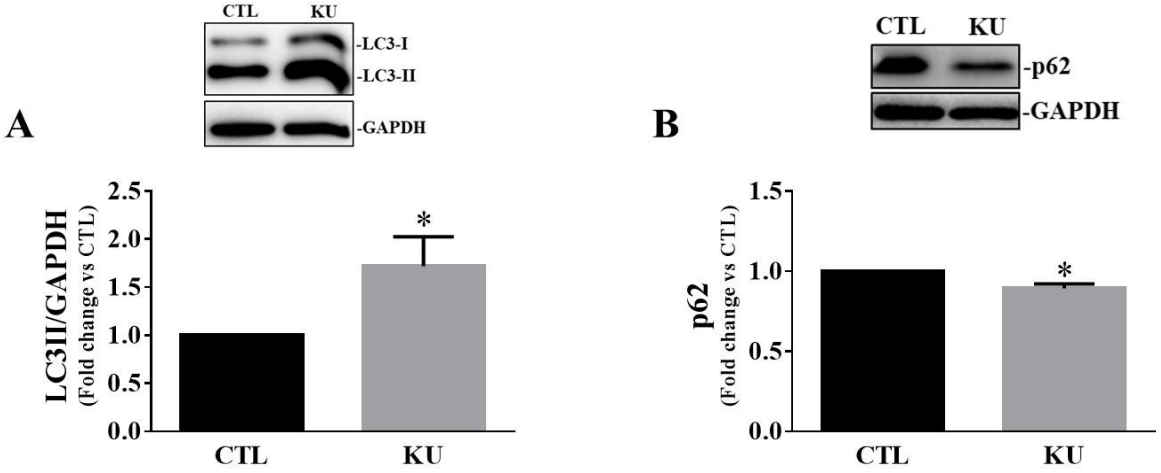


Figure 3.5. LC3-II and p62 protein levels in cardiac fibroblasts. Serum-starved cardiac fibroblasts were treated with KU-55933 (100 µm; ATM inhibitor) for 24 hours. Total cell lysates were analyzed by western blot using LC3B (A) and p62 (B) antibodies. Top: western blot exhibiting immunostaining for LC3-I, LC3-II, p62, and GAPDH. Bottom: quantitative analysis of LC3-II normalized to GAPDH (*p<0.05 vs CTL; *n=4*).

## Discussion

We have previously shown that ATM deficiency results in autophagic impairment early during MI (38). This is the first study investigating the role of ATM in cardiac autophagy in a chronic setting post-MI in mice. A major finding of this study is that ATM deficiency does not impair autophagic response in the non-infarct LV region 28 days post-MI. However, it augments autophagy in the infarct region via a different mechanism(s) than WT hearts. MI augments autophagy in the infarct regions of both WT and hKO hearts as evidenced by increased LC3-II and Beclin protein levels and decreased p62 protein levels. However, changes in autophagy may be more pronounced during ATM deficiency as evidenced by increased protein levels of LC3-II

and cathepsin D. Furthermore, autophagic changes in WT hearts post-MI associates with enhanced AMPK activation, while autophagic changes in hKO hearts post-MI associates with enhanced Akt and mTOR activation. ERK1/2 activation was increased to a similar extent in both MI groups. *In vitro*, ATM inhibition for 24 hours resulted in altered autophagic response in cardiac fibroblasts as evidenced by an increase in LC3-II protein levels coupled with a decrease in p62 protein levels.

Concurring with our previous finding that ATM alters autophagy at basal levels (38), LC3-II protein levels were significantly higher in ATM deficient hearts under basal conditions. However, there were no significant changes in Beclin, p62, or cathepsin D protein levels or signaling molecules related to autophagy in ATM deficient hearts. In our previous study, p62 protein levels were higher, while mTOR phosphorylation was lower in ATM deficient hearts under basal conditions (38). The reasons for these discrepant findings may include the age of the animals. In our previous study, the animals were ~4 months of age, while the mice in the current study are ~5 months of age. Autophagy is a dynamic process (27). It is possible that autophagic flux may change with age. However, further investigations are needed to explore this possibility.

Although autophagy is typically upregulated post-MI, studies have shown that autophagic activity often fluctuates throughout the duration of MI (16, 43). While it has been shown that autophagy is sharply induced 12-24 hours post-MI in the infarct boarder zone, it has also been shown to decrease 5-21 days post-MI in that same region (43). However, autophagosomes have been detected in surviving cardiomyocytes in chronic stages of MI, connecting autophagy to cardiac myocyte survival during the later stages of MI (21). We have previously shown that MI results in autophagic induction 4 hours during MI in WT hearts as evidenced by high LC3-II

protein levels coupled with reduced aggregate accumulation (38). Furthermore, phosphorylation of Erk1/2 was also higher 4 hours during MI in WT hearts (38). In the present study, MI resulted in autophagic augmentation 28 days post-MI in the infarct region as evidenced by an increase in both LC3-II and Beclin protein levels coupled with a decrease in p62 protein levels. Interestingly, phosphorylation of Erk1/2 and AMPK were significantly higher in the infarct region of WT hearts post-MI. Both AMPK and Erk1/2 have been implicated in the regulation of autophagy, typically acting as positive regulators of autophagic activity. Intermedin, a proopiomelanocortin-derived peptide, attenuated MI injury through the activation of autophagy in a rat model of ischemic heart failure via the activation of MAPK/ERK1/2 pathways (40). The compound curcumin induced autophagy in malignant glioma cells by activating Erk1/2 (32). Furthermore, inhibition of the Erk1/2 pathway inhibited curcumin-induced autophagy and apoptosis (32). AMPK, the energy sensor in cells, is a key player in the heart's response to stress (e.g. ischemia) (30). Under glucose starvation, AMPK promotes autophagy by directly activating Ulk1 via the phosphorylation of Ser 317 and Ser 777 (2, 18). Genetic inhibition of AMPK in cardiac myocytes attenuates cardiac autophagy, exacerbates cardiac dysfunction, and increases mortality in diabetic mice (44). Although Erk1/2 and AMPK signaling pathways have independently been shown to activate autophagy, there is evidence to suggest that an interplay between the two pathways may play an important role in the regulation of autophagy. Porcine circovirus type 2 induces autophagy in PK-15 cell via AMPK/Erk1/2 signaling (47). Thus, it is possible that the augmentation of autophagy in the infarct region 28 days post-MI in WT hearts may be due to Erk1/2 and AMPK signaling acting independently or in conjunction.

Similar to WT hearts post-MI, there was a significant increase in LC3-II and Beclin protein levels 28 days post-MI in the infarct region of ATM deficient hearts coupled with a

decrease in p62 protein levels. Together, these results suggest that MI alters autophagy in the infarct region of ATM deficient hearts 28 days post-MI as well. However, the mechanism(s) by which autophagy is augmented in ATM deficient hearts appear to be different than that of WT hearts. Although Erk1/2 activation was increased to a similar extent in both genotypes post-MI, activation of Akt and mTOR was higher in ATM deficient hearts post-MI. Activation of Akt and Erk1/2 is shown to positively regulate autophagy (35), while activation of mTOR is known to negatively regulate autophagy (7). Thus, it is possible that the autophagic changes in ATM deficient hearts post-MI involves both Akt and Erk1/2 signaling that is independent of mTOR activation.

Previously we have provided evidence that cardiac cell apoptosis is lower in the infarct region of ATM deficient hearts when compared to their WT counterparts 28 days post-MI (10). This decrease in apoptosis associated with decreased activity of GSK-3β, a pro-apoptotic kinase (10). Activation of Akt is known to phosphorylate and inactivate GSK-3β (11). Consistent with these observations, we observed enhanced activation of Akt in ATM deficient hearts. ATM deficient hearts also exhibited higher levels of LC3-II and cathepsin D protein in the infarct region indicating augmented autophagy during ATM deficiency post-MI. Inhibition of GSK-3β using CHIR99021 induced an autophagic response in human pancreatic cancer cells (24) and knockdown of GSK-3β increased basal autophagy in human aortic endothelial cells (41). Thus, it is possible that the alterations in autophagy in the infarct region of ATM deficient is due to inactivation of GSK-3β (phosphorylation) despite a decrease in AMPK activity and increase in mTOR activity. Together, these data provide evidence for a link between Akt/GSK-3β pathway, apoptosis, and autophagy in the infarct region of ATM deficient hearts 28 days post-MI. It is

possible that cardiac cell apoptosis is lower in ATM deficient hearts since autophagy is altered in response to activation of Akt and inactivation of GSK-3β.

Fibroblasts play a central role in post-MI repair and remodeling. In fact, fibroblasts constitute the majority of cells in the infarcted area during the infarct healing phase post-MI (36). Treatment of adult cardiac fibroblasts with the ATM inhibitor KU-55933 for 24 hours resulted in autophagic changes as evidenced by an increase in LC3-II protein levels and a decrease in p62 protein levels. In our previous study, we provided evidence that treatment of cardiac fibroblasts with ATM inhibitor KU-55933 for 4 hours results in autophagic impairment in adult cardiac fibroblasts as levels of LC3-II protein were lower in KU-treated cells (38). Together, these results provide evidence that autophagic activity changes over time in cardiac fibroblasts in response to ATM inhibition, an observation similar to ATM deficient hearts post-MI. The autophagic changes seen 4 hours post ATM inhibition (38) are different from those seen 24 hours post ATM inhibition.

In summary, both WT and hKO hearts exhibited augmented autophagy in the infarct regions, not the non-infarct region, 28 days post-MI. Autophagic changes are more pronounced in ATM deficient hearts post-MI as well as in cardiac fibroblasts treated with ATM inhibitor. The signaling molecules involved in autophagic changes appeared different between the two genotypes. Autophagy changes in WT hearts post-MI may involve the activation of AMPK and Erk1/2, while autophagy changes in hKO hearts post-MI involves activation of Akt and Erk1/2 that appears to be mTOR independent. Autophagy regulation in ATM deficient hearts may also occur via the involvement of Akt-mediated inactivation of GSK-3β. It should be emphasized that our collective data investigating the role of ATM in cardiac autophagy are obtained 4 hours post-

MI (38) or 28 days post-MI. Intermediate time points between 4 hours and 28 days post-MI may be helpful to understand the full scope of molecular changes involved in autophagic flux during ATM deficiency post-MI.


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## Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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CHAPTER 4

# LACK OF ATAXIA-TELANGIECTASIA MUTATED KINASE ALTERS GLUCOSE AND TRICARBOXYLIC ACID CYCLE METABOLISM IN THE HEART

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**Running title:** ATM deficiency and glucose metabolism in the heart

**Total number of figures:** 7

**Key words:** ATM, glucose, TCA cycle, metabolism, ischemia

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Abstract

Abnormalities in glucose metabolism are implicated in the progression of heart failure. Ataxia-telangiectasia mutated kinase (ATM) is a cell cycle checkpoint protein activated in response to DNA damage. Mutations in ATM cause a multisystemic disease known as Ataxia-telangiectasia (AT) that is often accompanied by glucose intolerance. Previous work from our lab has shown that lack of ATM induces structural and functional changes in the heart. Here we tested the hypothesis that lack of ATM alters cardiac glucose and TCA cycle metabolism with or without ischemia. Isolated wild-type (WT) and ATM knockout (KO) hearts underwent retrograde perfusion through the aorta using KH buffer containing U-$^{13}C_6$ glucose for 30 minutes. A separate group of hearts also underwent global ischemia for 20 minutes. Analyses of metabolome reservoir for the incorporation of $^{13}C$ using nuclear magnetic resonance (NMR) spectroscopy and ion chromatography mass spectrometry (IC-MS) revealed that lack of ATM accelerates glycolysis and gluconeogenesis in non-ischemic hearts in addition to augmenting TCA cycle metabolism. Global ischemia augments the gluconeogenic pathway in WT hearts, while it augments the glycolytic pathway in KO hearts that is associated with TCA cycle changes. Additionally, global ischemia has no effect on glycolytic or gluconeogenic pathways with minimal effects on TCA cycle metabolism in KO hearts compared to WT hearts. Phosphorylation of AMPK decreased post-ischemia in both WT and KO hearts, while it increased in KO hearts under non-ischemic and ischemic conditions compared to WT hearts. Glut4 protein expression decreased in KO hearts when compared to KO non-ischemic and WT ischemic hearts. Activation of Akt was lower, while activation of GSK-3β was higher post-ischemia in WT and KO hearts. Non-ischemic KO hearts also exhibited lower Akt activation and higher GSK-3β activation when compared to WT non-ischemic hearts. Thus, ATM affects

cardiac metabolites with and without ischemia which associated with changes in activation of Akt, GSK-3β, and AMPK alongside alterations in Glut4 protein levels.

## Introduction

Heart failure is the leading cause of death and disability globally (28, 35) and is estimated to increase 25% by 2030 (35). Heart failure is a complex syndrome that results from a plethora of diseases, ranging from diabetes, hypertension, and ischemic heart disease (35). However, a common thread that runs through the various etiologies leading to heart failure is modified cardiac metabolism (35). Recent studies reveal that metabolic remodeling is a prominent feature of heart failure (28, 35). Thus, metabolic therapy has risen as a promising approach to treating heart disease (32).

Under normal conditions or normoxia, glycolysis and the tricarboxylic acid **(**TCA) cycle contribute roughly 5% of the total ATP produced (11, 27, 28). Ischemia is associated with dramatic metabolic abnormalities in the heart, causing changes to organ function and even eventual heart failure (1, 27). One of the modifications that occurs in cells under ischemic conditions is the switch from aerobic to anaerobic metabolism (1), resulting in the predominant use of carbohydrates like glucose as an energy substrate (1, 27). Glucose uptake and glycolysis are accelerated in an effort to maintain myocardial energetics during myocardial ischemia (10, 27, 28, 32). In fact, increased glucose metabolism has been implicated in the progression of heart failure as it is upregulated in hypertrophied hearts (22). Dysfunction or depletion of the TCA cycle results in a decline in cardiac function (19). In addition to glycolysis, gluconeogenesis, another carbohydrate metabolism pathway, is also critical in the maintenance of cell energy homeostasis under normal and ischemic conditions (1). Although predominantly shown in liver, gluconeogenesis has been shown to increase in the rabbit heart under non-ischemic and ischemic conditions (1).

Ataxia-telangiectasia mutated kinase (ATM), a serine/threonine kinase, is generally activated in response to oxidative stress and DNA damage. It plays a critical role in cell cycle arrest and DNA repair. Although primarily located in the nucleus, it is found in the cytoplasm where it is suggested to play a pivotal role in metabolic pathways (7). It is suggested to modulate carbon metabolism in cancer cells (7, 24, 31). Deficiency of ATM affects metabolic parameters in mice (31). $ATM^{+/-}$ mice fed a western diet were glucose intolerant and insulin resistant compared to $ATM^{+/+}$ mice (31). Furthermore, $ATM^{+/-}$ mice had higher systolic and diastolic blood pressure (31). Mutations in the *ATM* gene cause an immunodeficiency and neuronal degeneration disease called Ataxia-telangiectasia (AT), a disorder that affects 1:40,000-1:100,000 people worldwide (7). AT patients exhibit many characteristics of metabolic disease, including susceptibility to diabetes and impaired glucose metabolism (33).

Previous work from our lab has shown that lack of ATM induces structural and functional parameters of the heart (18). We have also provided evidence that ATM plays an important role in myocyte apoptosis and myocardial remodeling following myocardial infarction and β-adrenergic receptor stimulation (8, 9, 16, 17). Here, we tested the hypothesis that lack of ATM alters cardiac glucose and TCA cycle metabolism pre- and post-myocardial ischemia. The data presented here suggest that lack of ATM alters glucose and TCA cycle metabolism in the heart before and after global ischemia that associates with changes in the activation of AMPK, Akt, GSK-3β and Glut4 protein levels.

## Materials and Methods

### Vertebrate Animals

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the experiments were performed in accordance with the protocols approved by the East Tennessee State University Committee on Animal Care. ATM heterozygous mice (129xblack Swiss hybrid background), generated as described(3), were purchased from Jackson Laboratory. ATM homozygous knockout (KO) mice were generated by breeding ATM heterozygous mice. Genotyping was performed by PCR using primers suggested by the Jackson Laboratory. Age-matched (~ 2-month-old) male and female (WT and KO) mice were used for the study.

### Langendorff perfusion of heart with $^{13}C_6$-glucose

Hearts were excised from WT and KO mice and briefly washed in PBS to remove excess blood. Hearts were then immediately perfused retrogradely with KH buffer containing U-$^{13}C_6$ glucose [(in mmol/L) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 24.8 $NaCHO_3$, 2.5 $CaCl_2$, and 10.6 U-$^{13}C_6$ glucose] for 30 minutes as previously described (38). The KH buffer was maintained at 37°C and equilibrated with 95% oxygen and 5% carbon dioxide. Flow rate was measured using the Transonic Systems Inc. T106 Small Animal Flow Meter. There was no significant difference in flow rate between WT and KO hearts (data not shown).

Quenching and extraction of polar compounds, lipids, and proteins

At the end of the 30 minute period, a group of hearts were subjected to 20 minutes of global ischemia. Tissue extraction was performed as previously described (13). Briefly, hearts were snap frozen and ground into powder using liquid nitrogen. Roughly 20 mg of tissue powder was placed into a 15 mL polypropylene conical centrifuge tube and shaken vigorously in a mixture of chilled acetonitrile (2 mL), nanopore water (1.5 mL), and HPLC-grade chloroform (1 mL). The mixture was centrifuged at 3500xg for 20 minutes at room temperature to separate the polar (top), tissue debris (primarily denatured proteins; middle), and lipid (bottom) layers. The polar and lipid layers were collected, and the tissue debris layer was further extracted using 0.5 mL of chloroform: methanol: butylated hydroxytoluene (BHT) (2:1:1 ratio). The tissue debris layer, collected in a 1.5 microfuge tube, was centrifuged at 14,000xg for 20 minutes at 4°C to separate the three phases again. The extracted polar and lipid fractions were pooled with their main fractions. The polar and tissue debris fractions were lyophilized overnight, and the dry weight of tissue debris was used to normalize the metabolic content. The polar extracts were redissolved in 100% $D_2O$ comprised of 30 nmol perdeuterated 2,2′-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical shift and concentration reference for Nuclear Magnetic Resonance (NMR) measurement.

Determination of cardiac metabolites incorporated with 13C

Lyophilized samples from both WT and KO hearts were analyzed using Ion Chromatography Mass Spectrometry (IC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy to determine cardiac metabolites as previously described (13).

Preparation of myocardial tissue for western blot analysis

WT and KO hearts were perfused retrogradely with Krebs-Henseleit (KH) buffer [(in mmol/L) NaCl 118, $NaHCO_3$ 25, KCl 4.75, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $CaCl_2$ 1.9 and glucose 11.9] for 30 minutes. A group of the hearts as also subjected to 20 minutes of global ischemia. The hearts were then snap frozen in liquid nitrogen, pulverized, and suspended in RIPA buffer [10 mM Tris-HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride] with added protease inhibitors for protein analysis.

Western blot analysis

Equal amounts of heart lysates (25 μg) were resolved using SDS-PAGE. The proteins were then transferred to a PVDF membrane. The membrane was blocked for one hour using 5% nonfat milk and incubated overnight with primary antibodies against p-Akt (ser-473; 1:1000), p-GSK-3β (ser-9; 1:1000), Akt (1:1000), GSK-3β (1:1000), AMPK (1:1000) (Cell Signaling), p-AMPKα (1:1000), or Glut4 (1:1000) (Santa Cruz). Total protein immunostaining was used as a loading control for phosphorylated proteins, while GAPDH was used as a loading control for Glut4 protein. The immune-complexes were detected using appropriate secondary antibodies and chemiluminescent reagents. Protein signals were visualized using ImageQuant LAS 500 imager and quantified using ImageQuant TL software (GE).

Statistical analysis

Data are expressed as means ± SE. Data were analyzed using a two-way analysis of variance followed by the Student-Newman-Keuls test. P values of <0.05 were considered to be significant.

## Results

Glycolytic metabolites

Formation of glucose-6-phosphate (G6P) is the first reaction of glycolysis. G6P can then be converted into pyruvate through a multi-step process involving the formation of fructose-6-phosphate (F6P) and phosphoenolpyruvate (PEP). Alternatively, it can be converted into glucose-1-phosphate (G1P) for conversion into glycogen. Using $^{13}C_6$*-glucose (+6)* isotopomer perfusion in isolated hearts, we observed significantly higher levels of six labelled carbon metabolites (+6 G1P, G6P and F6P; Fig 4.1A-C) and three labelled carbon metabolites (Fig 4.1D and 4.1F) in ATM deficient hearts versus WT (Fig 4.1A-C). The levels of labelled metabolites was ~4.8, 9.7, 7.0, 4.6 and 2.7 fold higher for G6P, F6P, G1P, PEP and pyruvate, respectively, in ATM deficient hearts vs WT without ischemia. Global ischemia (20 minutes) did not change levels of G6P, F6P and G1P in WT group. However, the levels of three carbon labelled metabolites, PEP and pyruvate, were significantly lower in WT-ischemic group vs WT-non-ischemic group. On the other hand, global ischemia in ATM KO hearts led to a significant decrease in all five metabolites vs KO-non-ischemic group (Fig 4.1A-1E). In fact, there was no significant difference in the levels of six or three carbon metabolites between the two ischemic groups.

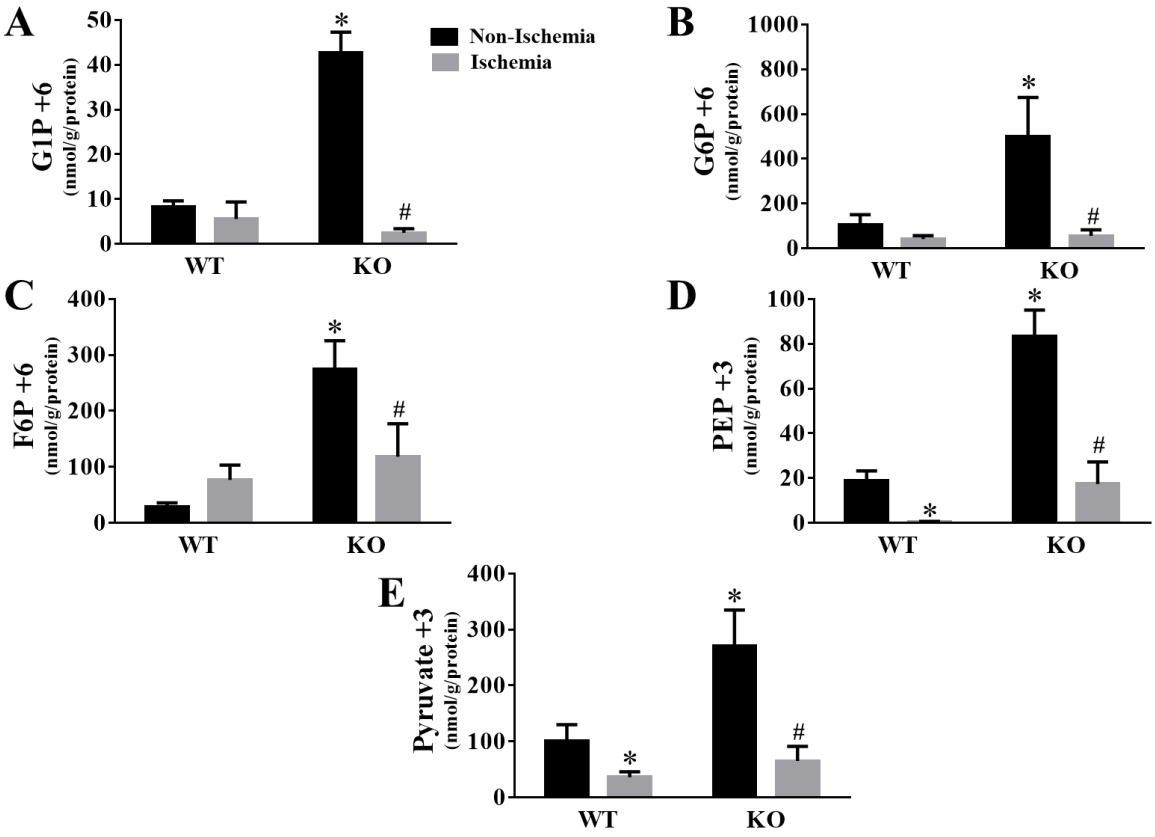


Figure 4.1. Glycolytic metabolites. Levels of glycolytic metabolites were measured in WT and KO hearts 20 minutes post-ischemia using Ion Chromatography Mass Spectrometry (IC-MS). *A:* G1P (+6). *B:* G6P (+6). *C:* F6P (+6). *D:* PEP (+3). *E:* Pyruvate (+3). *p<0.05 vs. WT-non; #p<0.05 vs. KO-non; n=3-7. G1P, Glucose-1-Phosphate; G6P, Glucose-6-Phosphate; F6P, Fructose-6-Phosphate; PEP, Phosphoenolpyruvate. (+n) represents the number of $^{13}$C carbons incorporated into that metabolite.

Gluconeogenic metabolites

Using *$^{13}C_6$-glucose (+6)* isotopomer perfusion in isolated hearts, we observed significantly higher levels of three carbon labelled (+3) metabolites, G1P and F6P, in ATM deficient hearts versus WT (Fig 4.2A, 4.2C). Global ischemia (20 minutes) increased the levels of three carbon labelled metabolites G1P, G6P, and F6P in WT group. However, global ischemia did not change levels of three carbon labelled metabolites (G1P, G6P, and F6P) in KO-ischemic

group vs KO-non-ischemic group (Fig 4.2A-4.2C). There was no significant difference in the levels of three carbon labelled metabolites between the two ischemic groups.

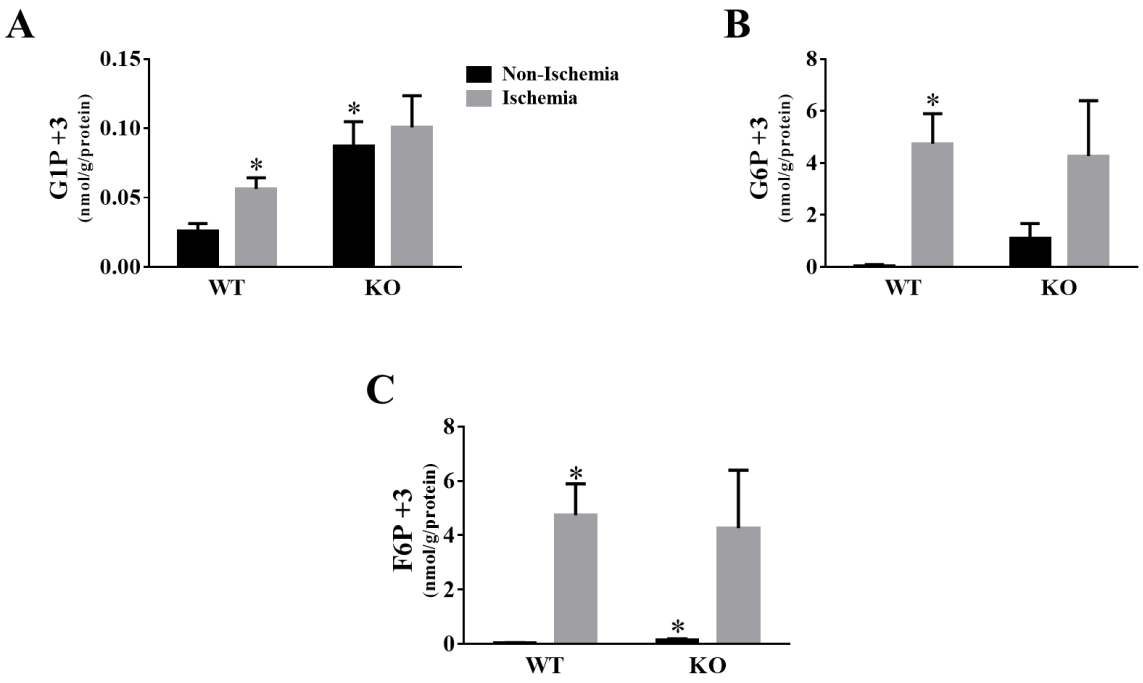


Figure 4.2. Gluconeogenic metabolites. Levels of gluconeogenic metabolites were measured in WT and KO hearts 20 minutes post-ischemia using IC-MS. *A:* G1P (+3). *B:* G6P (+3). *C:* F6P (+3). *p<0.05 vs. WT-non; n=3-7. G1P, Glucose-1-Phosphate; G6P, Glucose-6-Phosphate; F6P, Fructose-6-Phosphate. (+n) represents the number of $^{13}C$ carbons incorporated into that metabolite.

Glycogen and Lactate levels

There was no difference in glycogen levels between WT and KO hearts under non-ischemic Glycogen levels were significantly decreased post-ischemia in both groups with no difference between the two genotypes (WT-non, 9.27±1.69 nmol/mg; WT-isch, 1.16±0.41* nmol/mg; KO-non, 8.81±1.84 nmol/mg; KO-isch, 0.50±0.34$^{\#}$ nmol/mg; *p<0.05 vs. WT-non; $^{\#}$p<0.05 vs. KO-non; n=3-5; Figure 4.3A). There were no significant changes in lactate (+3) levels in the heart (WT-non, 1059864.79±653274.58 nmol/g/protein; WT-isch,

2393917.12±1125072.61 nmol/g/protein; KO-non, 2630613.33±1606850.02 nmol/g/protein; KO-isch, 5801752.79±2585945.95 nmol/g/protein; n=5-7; Figure 4.3B).

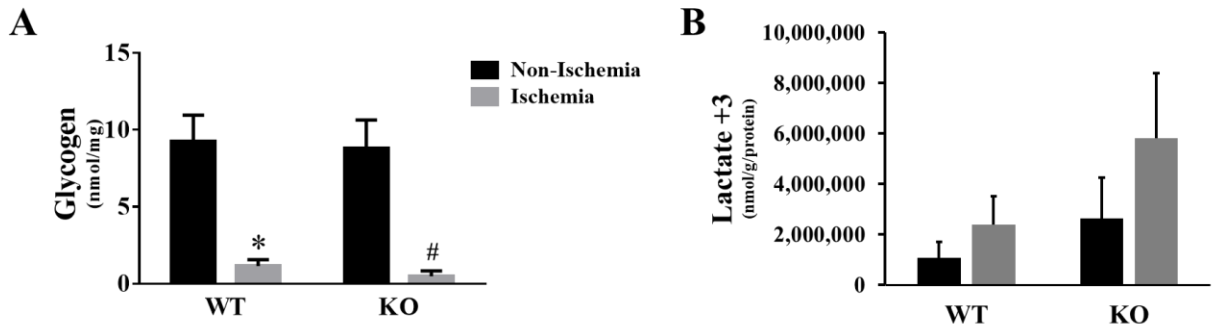


Figure 4.3. Glycogen and Lactate levels. Glycogen levels were measured in WT and KO hearts 20 minutes post-ischemia using Nuclear Magnetic Resonance (NMR) spectroscopy, while lactate levels were measured in those hearts 20 minutes post-ischemia using IC-MS. *p<0.05 vs. WT-non; $^{\#}$p<0.05 vs. KO-non; n=3-7. (+n) represents the number of $^{13}$C carbons incorporated into that metabolite.

TCA cycle metabolites

Citrate levels

Citrate levels were not different between the two non-ischemic groups. Global ischemia decreased citrate levels to a similar extent in both groups (WT-non, 320.12±134.82 nmol/g/protein; WT-isch, 40.42±9.18* nmol/g/protein; KO-non, 236.70±49.32 nmol/g/protein; KO-isch, 104.37±40.19$^{\#}$ nmol/g/protein; *p<0.05 vs. WT-non; $^{\#}$p<0.05 vs. KO-non; n=4-6; Figure 4.4A)

Isocitrate levels

Isocitrate levels were significantly lower in KO hearts under non-ischemic conditions compared to WT hearts. Global ischemia led to a significant decrease in isocitrate levels in WT

hearts compared to their non-ischemic counterpart. Isocitrate levels remained unchanged in KO hearts compared to their non-ischemic counterpart with no significant difference between the two genotypes post-ischemia (WT-non, 147.89±51.30 nmol/g/protein; WT-isch, 5.48±2.70* nmol/g/protein; KO-non, 23.43±18.72* nmol/g/protein; KO-isch, 19.89±12.93 nmol/g/protein; *$p<0.05$ vs. WT-non; n=4-6; Figure 4.4B).

α-ketoglutarate levels

α-ketoglutarate levels were significantly higher in KO hearts under non-ischemic conditions compared to WT hearts. Global ischemia decreased α-ketoglutarate levels in both groups with no significant difference between the two genotypes post-ischemia (WT-non, 1.20±0.27 nmol/g/protein; WT-isch, 0.23±0.11* nmol/g/protein; KO-non, 6.56±1.06* nmol/g/protein; KO-isch, 0.15±0.06$^{\#}$ nmol/g/protein; *$p<0.05$ vs. WT-non; $^{\#}p<0.05$ vs. KO-non; n=3-7; Figure 4.4C).

Succinate levels

Succinate levels were significantly higher in KO hearts under non-ischemic conditions compared to WT hearts. Global ischemia increased succinate levels significantly in WT hearts, not in KO hearts (WT-non, 7.82±1.83 nmol/g/protein; WT-isch, 331.31±59.67* nmol/g/protein; KO-non, 37.94±4.84* nmol/g/protein; KO-isch, 86.71±34.26$^{\$}$ nmol/g/protein; *$p<0.05$ vs. WT-non; $^{\$}p<0.05$ vs. WT-isch; n=3; Figure 4.4D).

Fumarate levels

Fumarate levels were significantly higher in KO hearts under non-ischemic conditions compared to WT hearts. Global ischemia increased fumarate levels in both genotypes with no difference between the two groups (WT-non, 49.81±13.77 nmol/g/protein; WT-isch, 204.89±53.14* nmol/g/protein; KO-non, 99.06±14.45* nmol/g/protein; KO-isch, 260.17±26.55$^{\#}$ nmol/g/protein; *$p<0.05$ vs. WT-non; $^{\#}p<0.05$ vs. KO-non; n=4-6; Figure 4.4E).

Malate levels

Malate levels were not different between the two genotypes under non-ischemic conditions. Malate levels remained unchanged between the two groups post-ischemia when compared to their respective non-ischemic group. However, malate levels were significantly higher post-ischemia in KO hearts compared to WT hearts (WT-non, 110.99±34.93 nmol/g/protein; WT-isch, 96.39±13.59 nmol/g/protein; KO-non, 206.01±102.89 nmol/g/protein; KO-isch, 221.62±36.31$^{\$}$ nmol/g/protein; $^{\$}p<0.05$ vs. WT-isch; n=5-7; Figure 4.4F).

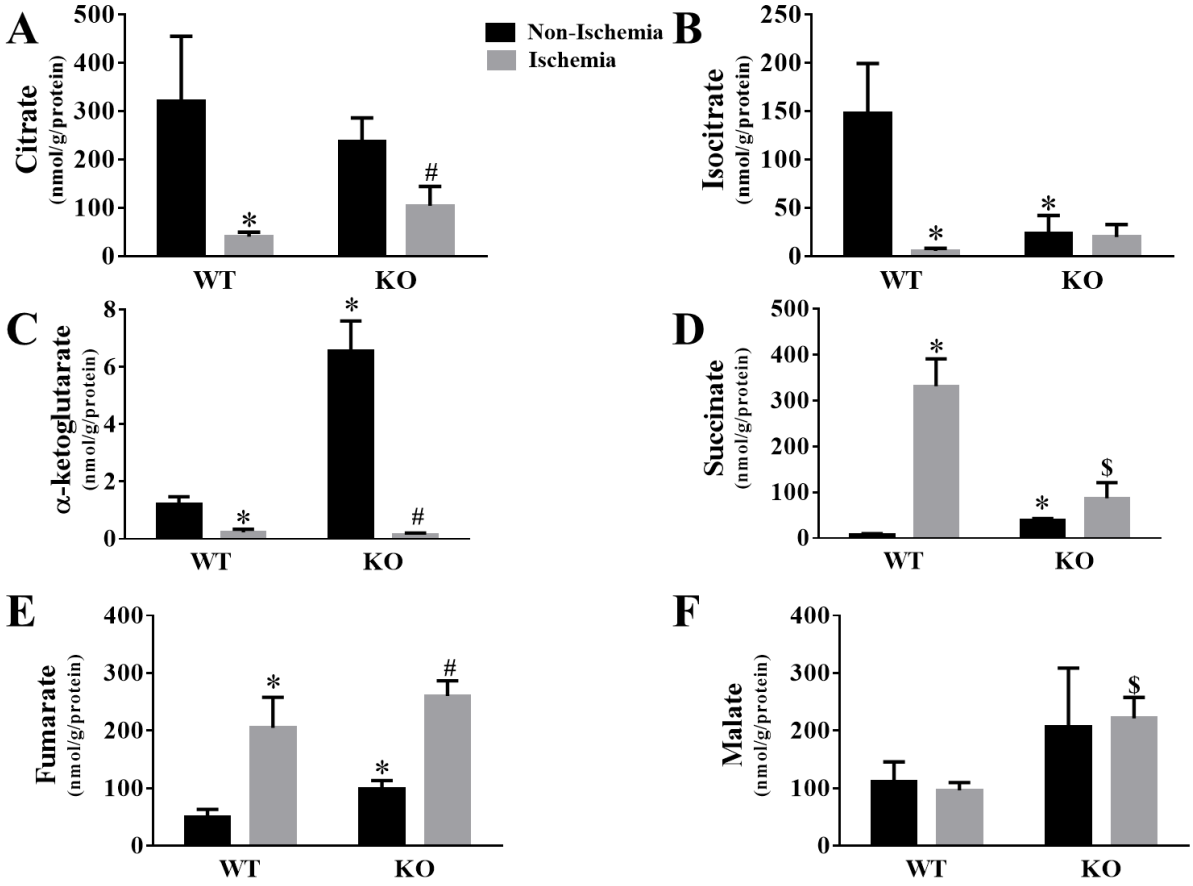


Figure 4.4. TCA cycle metabolites. Levels of TCA cycle metabolites were measured in WT and KO hearts 20 minutes post-ischemia using IC-MS. *A:* Citrate. *B:* Isocitrate. *C:* α-ketoglutarate. *D:* Succinate. *E:* Fumarate. *F:* Malate. *p<0.05 vs. WT-non; $^{\#}$p<0.05 vs. KO-non; $^{\$}$p<0.05 vs. WT-isch; n=3-7. All (+n) metabolite data was pooled to yield total TCA cycle metabolite levels.

Aspartate and Glutamate levels

Aspartate and glutamate levels remained unchanged among the four groups (data not shown).

ATP levels

ATP levels were significantly higher in WT hearts under non-ischemic conditions compared to KO hearts. Global ischemia significantly decreased ATP levels in WT hearts

compared to their non-ischemic counterpart. ATP levels remained unchanged in KO hearts post ischemia (WT-non, 838.50±401.60 nmol/g/protein; WT-isch, 43.46±34.21* nmol/g/protein; KO-non, 8.07±5.79* nmol/g/protein; WT-isch, 1.07±0.58 nmol/g/protein; *p<0.05 vs. WT-non; n=3-7; Figure 4.5).

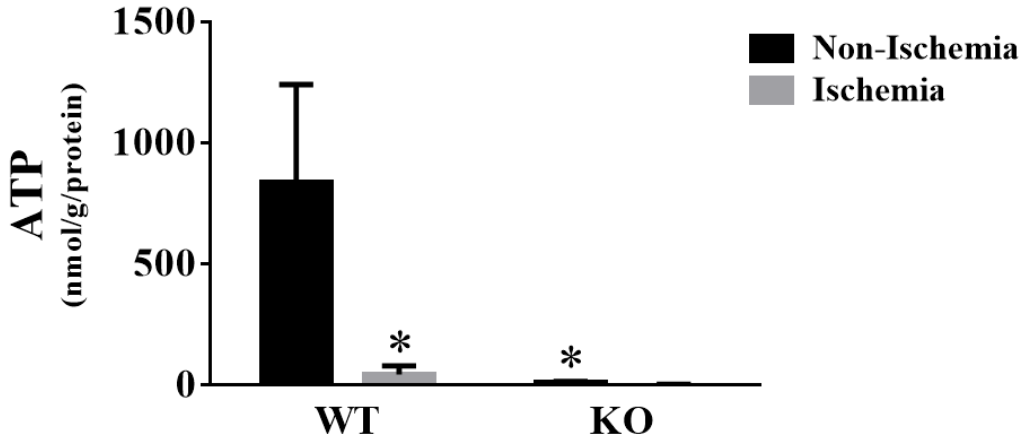


Figure 4.5. ATP levels. ATP levels were measured in WT and KO hearts 20 minutes post-ischemia using IC-MS. *p<0.05 vs. WT-non; n=3-7.

AMPK activation and Glut4 expression

Phosphorylation (activation) of AMPK was significantly higher in KO hearts under non-ischemic conditions compared to WT hearts. Global ischemia decreased activation of AMPK in both groups. However, phosphorylation of AMPK remained significantly higher in KO hearts compared to WT hearts post-ischemia (*p<0.05 vs. WT-non; $^{\#}$p<0.05 vs. KO-non; $^{\$}$p<0.05 vs. WT-isch; n=4-5; Figure 4.6A).

Glut4 protein levels were not different between the two genotypes under non-ischemic conditions. Global ischemia significantly decreased Glut4 levels in KO group, not in WT ($^{\#}p<0.05$ vs. KO-non; $^{\$}p<0.05$ vs. WT-isch; n=4-5; Figure 4.6B).

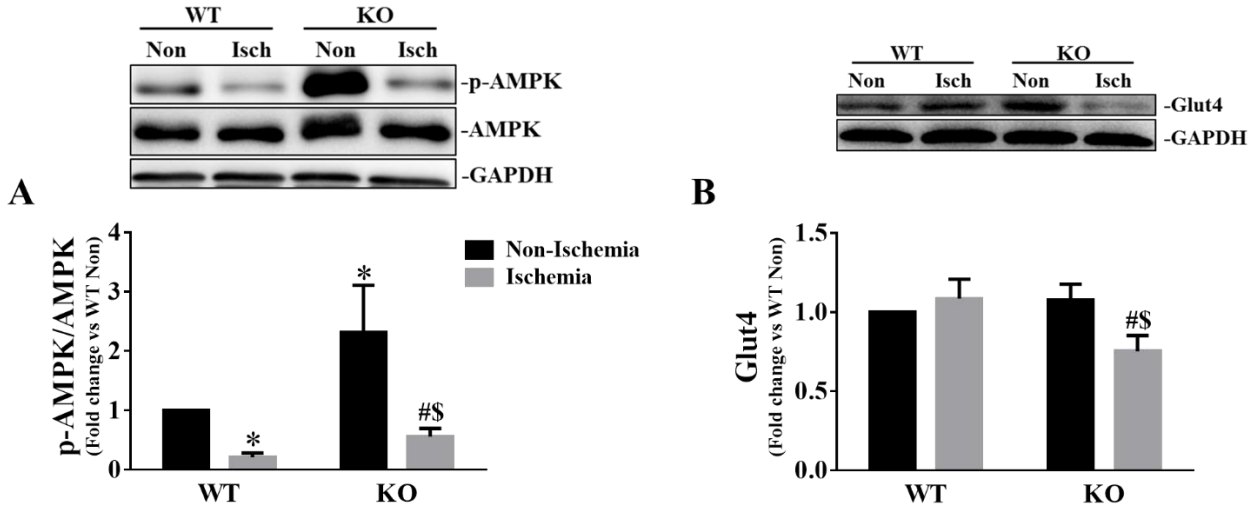


Figure 4.6. AMPK activation and Glut4 expression. Heart lysates, prepared from WT and KO hearts, were analyzed by Western blot using anti-p-AMPK (A) and Glut4 (B) antibodies. Top: western blots exhibiting immunostaining for p-AMPK, Glut4, and GAPDH. Bottom: quantitative analyses of p-AMPK normalized to AMPK and Glut4 normalized to GAPDH (*$p<0.05$ vs. WT-non; $^{\#}p<0.05$ vs. KO-non; $^{\$}p<0.05$ vs. WT-isch; n=4-5).

Akt and GSK-3β activation

Phosphorylation (activation) of Akt was significantly higher in WT hearts under non-ischemic conditions compared to WT hearts. Global ischemia significantly decreased Akt phosphorylation in both groups when compared to their respective non-ischemic group. There was no statistical difference in Akt phosphorylation between the two genotypes post-ischemia (*$p<0.05$ vs. WT-non; $^{\#}p<0.05$ vs. KO-non; n=5; Figure 4.7A).

Phosphorylation at Ser-9 inactivates GSK-3β (14). Phosphorylation of GSK-3β was significantly lower in KO hearts under non-ischemic conditions compared to the WT hearts.

Global ischemia decreased GSK-3β phosphorylation on both groups when compared to their respective non-ischemic group. There was no statistical difference in GSK-3β phosphorylation between the two genotypes post-ischemia (*p<0.05 vs. WT-non; #p<0.05 vs. KO-non; n=5; Figure 4.7B).

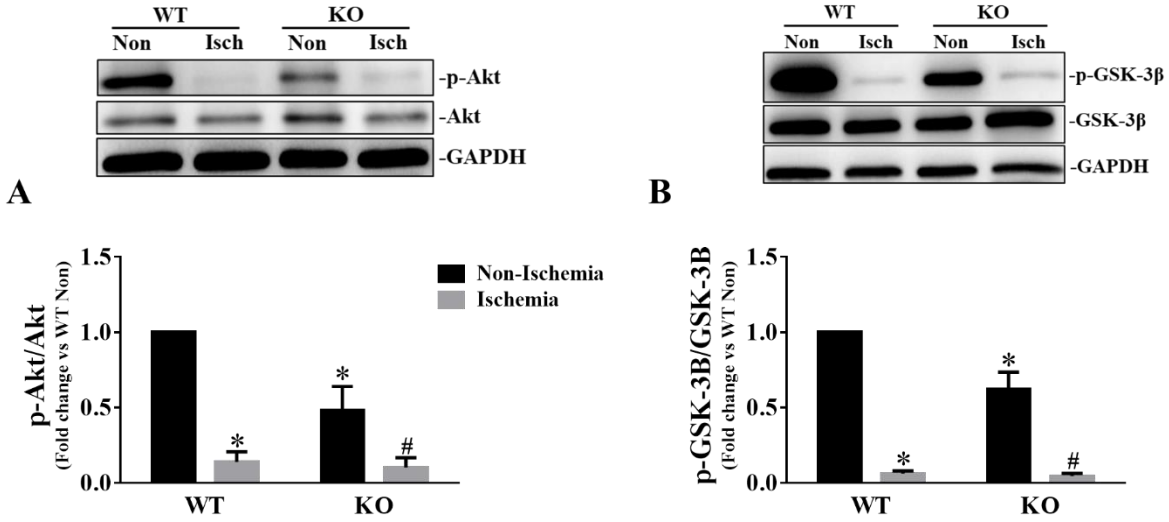


Figure 4.7. Akt and GSK-3β activation. Heart lysates, prepared from WT and KO hearts, were analyzed by Western blot using anti-p-Akt (A) and anti-p-GSK-3β (B) antibodies. Top: western blots exhibiting immunostaining for p-Akt, p-GSK-3β, and GAPDH. Bottom: quantitative analyses of p-Akt and p-GSK-3β normalized to Akt and GSK-3β (*p<0.05 vs. WT-non; #p<0.05 vs. KO-non; n=5).

## Discussion

A major finding of this study is that the lack of ATM accelerates glycolysis and gluconeogenesis and augments TCA cycle metabolism in the heart under non-ischemic conditions. Global ischemia augmented the gluconeogenic pathway in WT hearts, while it augmented the glycolytic pathway during ATM deficiency which associated with changes in TCA cycle metabolites. ATP levels were significantly lower in ATM deficient hearts. Global

ischemia decreased ATP levels in both genotypes. These metabolic changes associated with alterations in the activity of AMPK, Akt, and GSK-3β as well as protein levels of Glut4.

AT patients, patients with mutations in *ATM* gene, are predisposed to breast and pancreatic cancer and ischemic heart disease. These patients also show enhanced susceptibility to diabetes and impaired glucose metabolism (7, 33). In fact, AT patients exhibit lower metabolism in cerebellar hemisphere, anterior vermis and fusiform, while exhibiting higher metabolism in globus pallidus (34). This decreased metabolism in globus pallidus correlated negatively with motor performance (34). Previously, our lab provided evidence that lack of ATM induces structural and functional changes in the heart with enhanced cardiac fibrosis and hypertrophy (18). Here we observed that lack of ATM results in enhanced glycolysis and gluconeogenesis at under non-ischemic conditions as evidenced by an increase in glycolytic metabolites such as G1P (+6), G6P (+6), F6P (+6), PEP (+3), and pyruvate (+3), and gluconeogenic metabolites such as G1P (+3) and F6P (+3) when compared to WT hearts. Additionally, ATP levels were lower in ATM null hearts under non-ischemia. This is consistent with the observations of Zakikhani et al. where glucose uptake and glycolysis were higher, while ATP levels were lower in MCF-7 and HepG2 cells treated with an ATM inhibitor KU-55933 (40). It was interesting to note that lack of ATM had no effect on glycogen and lactate levels under non-ischemic conditions. The enhanced glycolysis in ATM null hearts suggests metabolic stress. It is possible that glycolysis is enhanced in KO hearts as an attempt to replenish ATP levels and maintain somewhat proper functioning of the heart. However, these efforts may be restrained by enhanced gluconeogenesis, another mode by which homeostasis could be restored. It is also possible that glycogen levels remained unchanged under non-ischemic conditions in KO hearts due to rapid cycling of both glycolytic and gluconeogenic pathways.

The metabolic demand of the heart per gram is the highest in the body (37). Adequate amount of ATP must be generated to support the contractile activity of the heart and to support viability (37). About 4% of ATP in the heart is derived from glycolysis, while >95% of ATP is derived from oxidative phosphorylation at the electron transport chain in mitochondria (37). Cardiac ischemia associates with dramatic changes in metabolism. Due to depletion of $O_2$, mitochondrial oxidative phosphorylation decreases, while glycolysis accelerates (27). Since mitochondria are unable to oxidize pyruvate, pyruvate is converted into lactate (27). As expected, global ischemia decreased ATP levels and glycogen levels in WT hearts. Global ischemia also associated with increased levels of gluconeogenic metabolites, such as G1P (+3), G6P (+3), and F6P (+3). The levels of glycolytic metabolites, PEP (+3) and pyruvate (+3), were significantly lower in ischemic WT hearts. Levels of lactate tended to be higher with ischemia, although the data remained insignificant. These data point towards the augmentation of the gluconeogenic pathway in WT hearts post-ischemia. Furthermore, the decrease in glycogen levels post-ischemia is indicative of glycogen breakdown. Glycogen is a critical energy repository in the heart and can quickly be mobilized if needed (35). During energy deficiency, glycogen breakdown is enhanced to accommodate the rapid changes in cardiac demand (35). In rat kidney, 30 minutes of ischemia led to enhanced gluconeogenesis (23). Gluconeogenesis is suggested to be correlated with glycogen metabolism in rabbit hearts during ischemia and reperfusion as ischemia (5 minutes) decreased glycogen levels and increased the expression of phosphoenolpyruvate carboxykinase, an important enzyme involved in the regulation of gluconeogenesis (1). Given that there is no significant change in lactate levels, and the levels of glycolytic metabolites PEP (+3) and pyruvate (+3) are lower in WT hearts post-ischemia, it is

possible that PEP (+3) and pyruvate (+3) contribute to the gluconeogenic pathway. It is also possible that glycogen is mobilized in WT hearts post-ischemia for gluconeogenesis.

In contrast to WT hearts, ATP levels were lower in KO hearts and remained unchanged post-ischemia. Interestingly, non-ischemic levels of glycolytic metabolites - G1P (+6), G6P (+6), F6P (+6), PEP (+3), and pyruvate (+3) were significantly greater in KO hearts vs WT hearts. Global ischemia decreased the levels of these metabolites with no difference between the two genotypes. Two gluconeogenic metabolites GIP (+3) and F6P (+3) were also greater in non-ischemic KO hearts and global ischemia had no effect on gluconeogenic metabolites. Like WT hearts, global ischemia decreased glycogen levels with no change in lactate levels. It appears that global ischemia augments the glycolytic pathway in KO hearts. The decrease in glycogen stores coupled with the decrease in glycolysis may indicate a preference for glycogen metabolism as opposed to metabolizing exogenous glucose in KO hearts post-ischemia.

Additionally, global ischemia decreased levels of citrate, isocitrate, and α-ketoglutarate, while increasing levels of succinate and fumarate. Since the TCA cycle is oxygen-dependent, the levels of TCA cycle metabolites citrate, isocitrate, and α-ketoglutarate are expected to be lower in WT hearts post-ischemia. Unexpectedly, ischemia elevated levels of succinate and fumarate in WT hearts. Succinate is suggested to play a critical role in the heart's response to ischemia-reperfusion injury, and is considered as a universal signature of ischemia (5, 30) as its accumulation has been seen during hypoxia in isolated adult rat heart cells (21) and in isolated mouse hearts (2). The sources of increased succinate can include the conversion of aspartate and glutamate to succinate (2) or reduction of malate to succinate via the reverse reaction of succinate dehydrogenase during ischemia (2, 30). Of note, accumulation of fumarate is suggested

to play a cardioprotective role (2). Therefore, it is possible that enhanced production of succinate and fumarate from amino acid metabolism may be an attempt to generate energy through intermediary metabolism, while playing a protective role to reverse/negate the negative consequences of cardiac ischemia.

KO mice also exhibited altered TCA cycle metabolism under non-ischemic conditions. Isocitrate levels were lower, while α-ketoglutarate, succinate, and fumarate levels were higher in KO hearts. The decrease in isocitrate levels could be due to the usage of pyruvate to fuel glycolytic and gluconeogenic cycling as opposed to being utilized to drive the TCA cycle. The increase in the levels of α-ketoglutarate, succinate, and fumarate may in part be attributable to anaplerosis of the TCA cycle. Amino acids play a critical role in cardiac metabolism, as they are regarded as cardioprotective substrates and produce low levels of acidic by-products (12). Glutamate, a key amino acid in cellular metabolism, can easily be converted to α-ketoglutarate in times of stress (12). Aspartate has the ability to remove excess TCA cycle intermediates downstream from the conversion to succinate (12). Here, glutamate and aspartate levels were not significantly different between the two non-ischemic groups. It is possible that substrates other than glutamate and aspartate may contribute to α-ketoglutarate formation in KO hearts. Global ischemia decreased citrate and α-ketoglutarate levels in both genotypes, however, fumarate levels were increased in KO hearts, indicating that the TCA cycle may be defective or down-regulated in KO hearts during global ischemia. Fumarate levels may be higher due to its potential cardio-protective effect. Despite the robust changes seen in KO hearts under non-ischemic conditions when compared to WT hearts, global ischemia had no effect on the glycolytic and gluconeogenic pathways in KO hearts. It appears that KO hearts are metabolically stressed without the superimposed insult.

AMPK is a key component of many metabolic pathways in the heart, as it serves to maintain cellular homeostasis (20, 26, 28, 39). AMPK is activated in response to an increase in the AMP:ATP ratio and metabolic stresses like ischemia and oxidative stress (20, 26, 28, 39). In fact, AMPK is a key regulator of glucose metabolism as its activation triggers catabolic pathways like glycolysis to produce ATP (20, 26, 28, 39), while turning off anabolic pathways that utilize ATP such as gluconeogenesis (26, 39). In order to increase glucose uptake, AMPK increases GLUT1 expression and GLUT4 translocation to the plasma membrane (20, 28, 32, 35). However, GLUT1 is the predominant glucose transporter in the fetal heart, while GLUT4 is the predominant form in the adult heart (10, 11). In fact, after birth GLUT4 replaces GLUT1 yielding a 20-fold decrease in GLUT1 and a 4-fold increase in GLUT4 mRNA and protein (10). While GLUT1 is important for basal glucose uptake in the fetal heart, GLUT4 mediates glucose uptake in the adult heart and is correlated with sustained cardiac function (10, 11). Here we observed that AMPK activity is higher in KO non-ischemic hearts. Global ischemia decreased AMPK activity in both genotypes. On the other hand, GLUT4 expression was not different between the two non-ischemic groups. Global ischemia decreased GLUT4 expression in KO group, not in WT group. The observation of higher AMPK activity in non-ischemic KO hearts is consistent with the observations Zakikhani et al. where AMPK phosphorylation was increased in MCF-7, HeLa, and HepG2 cells treated with the ATM inhibitor KU-55933 (40). This increased AMPK activation may be a response to low ATP levels in KO hearts under non-ischemic conditions. Although GLUT4 protein levels were not significantly different between WT and KO groups under non-ischemic conditions, high AMPK activation may explain the accelerated glycolysis. On the other hand, decreased AMPK activation in WT hearts post-ischemia may explain the increase in gluconeogenic metabolites to maintain homeostasis in the face of

ischemic stress, while AMPK activation in KO hearts post-ischemia may be correlated with low GLUT4 protein levels and glycolysis.

Akt signaling plays a key role in cell survival and glucose metabolism (4, 6, 36). In cancer cells, Akt signaling associates with an increase in glucose metabolism (6). Akt promotes glucose uptake by increasing GLUT1 expression on the cell surface and promoting the translocation of GLUT4 to the plasma membrane (4, 36). Akt also affects glucose metabolism by phosphorylating or inactivating GSK-3β, which in turn stimulates glycogen synthesis (25, 29). Ischemia increases GSK-3β transcription in rabbit hearts, followed by a decrease in glycogen content, suggesting that glycogen consumption during ischemia is regulated by GSK-3β (15). In the present study, non-ischemic KO hearts exhibited decreased Akt activation and increased GSK-3β activation versus the WT non-ischemic hearts. Previously, our lab reported no significant change in phosphorylation of Akt and GSK-3β between WT-sham and KO-sham groups (18). It should be noted that hearts in the present study were subjected to a 30 minute perfusion with KH buffer post-excision prior to the preparation of heart lysates. Furthermore, the previous study used LV lysates for western blot analyses (18), while the current study used whole heart lysates. Interestingly, glycogen levels were comparable between the two genotype, supporting the possibility of rapid cycling of both glycolytic and gluconeogenic pathways in KO hearts. Global ischemia enhanced GSK-3β activity, while decreasing Akt activity and glycogen levels. These data suggest that glycogen consumption is regulated by Akt/GSK-3β pathway in both genotypes post-ischemia. In the KO hearts, however, Akt activity may also be involved in Glut4 expression as Glut4 protein levels were lower in KO hearts post-ischemia as well.

In summary, we provide evidence that the lack of ATM accelerates glycolysis and gluconeogenesis in the heart during non-ischemic conditions to augment TCA cycle metabolism. Global ischemia augments the gluconeogenic pathway in WT hearts, while it augments the glycolytic pathway during ATM deficiency which associates with changes in TCA cycle metabolites. Additionally, global ischemia had no effect on glycolytic or gluconeogenic pathways in KO hearts, suggesting the possibility that the KO hearts are already metabolically stressed under non-ischemic conditions. The aforementioned metabolic changes associated with changes in the activity of signaling kinases such as Akt, GSK-3β, and AMPK, and Glut4 protein levels. Together, these data support the finding that ATM affects cardiac metabolites with and without ischemia. However, it should be emphasized that our data on ATM deficiency and metabolic changes are obtained 20 minutes post-global ischemia. A thorough time course analysis of various metabolites and signaling molecules involved metabolism may provide further insights into the mechanism by which ATM affects cardiac energetics.

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## Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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# CHAPTER 5

# CONCLUSION

Mutations in *ATM* gene cause a multisystemic disease known as Ataxia telangiectasia (AT) (Lavin et al. 1995; Su and Swift 2000). AT patients are susceptible to ischemic heart disease and metabolic disorders (Lavin et al. 1995; Su and Swift 2000). Using ATM deficient mice, this study investigated the role of ATM in cardiac autophagy and glucose metabolism under ischemic conditions. A major finding of this study is that ATM deficiency results in autophagic impairment in the heart 4 hours during MI and 4 hours post- inhibition in cardiac fibroblasts, while it augments autophagic response in the infarct region of the heart 28 days post-MI and 24 hours post-inhibition in cardiac fibroblasts. Early during MI (4 hours after its onset), ATM deficiency resulted in autophagic impairment in all phases (autophagy induction, autophagosome formation, and autophagolysosome degradation) and in cardiac fibroblasts via the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK. Late during MI (28 days post-MI), ATM deficiency augmented autophagy only in the infarcted region of the heart, which associated with the activation of Erk1/2, Akt and mTOR. Additionally, ATM affected glucose metabolism with and without ischemia. The lack of ATM accelerated glycolysis and gluconeogenesis, and augmented TCA cycle metabolism under non-ischemic conditions. Global ischemia augmented the glycolytic, not the gluconeogenic, pathway during ATM deficiency. Such metabolic changes associated with alterations in the activity of AMPK, Akt, and GSK-3β as well as protein levels of Glut4. Overall, the data presented here implicates ATM as a key player in autophagic changes in the heart in response to MI as well as in glucose metabolism under non-ischemic and ischemic conditions.

## ATM and Cardiac Autophagy

Early during MI (4 hours after its onset), ATM deficiency impaired autophagy in the heart at all three stages (autophagy induction, autophagosome formation, and autophagolysosome degradation) and in cardiac fibroblasts treated with the ATM inhibitor KU-55933 for 4 hours. Such autophagic changes in the heart and in cardiac fibroblasts associated with the activation of GSK-3β and mTOR, and inactivation of Akt and AMPK. Although typically associated with apoptotic signaling (Endo et al. 2006), Akt/ GSK-3β signaling has also been implicated in autophagy regulation. Akt activation (phosphorylation) and GSK-3β inactivation (phosphorylation) induces autophagy following middle cerebral artery occlusion that ultimately results in neuroprotection in the transient cerebral ischemic rat model (Qi et al. 2012). Furthermore, the GSK-3β inhibitor CHIR99021 induced an autophagic response in human pancreatic cancer cells (Marchand et al. 2015). Additionally, GSK-3β activity has been linked to AMPK activity, the energy sensor in cells that functions to maintain homeostasis by responding to ATP depletion. Suppression of GSK3β using CHIR99021 in human aortic endothelial cells resulted in increased basal autophagy and AMPK activity (Weikel et al. 2016). It is well-known that AMPK activation activates autophagy and can do so via inactivating the master regulator of autophagy, mTOR (Chang Hwa Jung, Seung-Hyun Ro, Jing Cao, Neil Michael Otto 2010; Kim et al. 2011; Kim and L. 2015). Thus, it is possible that inactivation of Akt leads to the activation of in GSK-3β phosphorylation, thus activating GSK-3β. Increased GSK-3β activity may result in a decline in AMPK phosphorylation and activation. This decline in AMPK activation may result in mTOR activation that ultimately inhibits autophagic response. Interestingly, ATM deficiency resulted in enhanced autophagic response under basal conditions. However, such autophagic changes did not associate with alterations in Akt, GSK-3β, or AMPK activity. However, mTOR

phosphorylation was lower in ATM deficient hearts, indicating that enhanced autophagic response in ATM deficient hearts under basal conditions may be mTOR dependent. Together, these data suggest that ATM deficiency serves a pro-autophagic role under basal conditions that changes upon the onset of MI, and that the signaling molecules involved in ATM-mediated autophagic response are different under basal conditions and early during MI.

ATM deficiency also augmented autophagy the infarct region of the heart 28 days post-MI as well as in cardiac fibroblasts treated with ATM inhibitor KU-55933 for 24 hours. Augmented autophagy in the infarct region of the hearts associated with enhanced Akt, Erk1/2, and mTOR activation. Activation of Akt and Erk1/2 is shown to positively regulate autophagy (Suffixidharan et al. 2011), while activation of mTOR is known to negatively regulate autophagy (Chang Hwa Jung, Seung-Hyun Ro, Jing Cao, Neil Michael Otto 2010). Thus, it is possible that the autophagic changes in ATM deficient hearts post-MI involves both Akt and Erk1/2 signaling that is independent of mTOR activation. Furthermore, we previously found that activation of GSK-3β was lower in the infarct region of ATM deficient hearts compared to their WT counterparts (Daniel et al. 2016). Of interest is to note that the decrease in GSK-3β activity associated with a decrease in apoptosis in the infarct region of the heart (Daniel et al. 2016). As GSK-3β is a downstream target of Akt (Fang et al. 2000), it is possible that activated Akt phosphorylated and inhibited (phosphorylated) GSK-3β. Such inhibition of GSK-3β, a pro-apoptotic kinase (Daniel et al. 2016), could have resulted in the decrease in apoptosis and changes in autophagy.

While autophagy is typically enhanced following MI, there have been contrasting reports of autophagic activity in the non-infarct region of the heart following MI. For instance, Chi et al.

showed that LC3 protein expression revealed that LC3 protein levels were significantly lower in the remote non-infarcted region of the rabbit hearts 1, 4 and 8 weeks after MI compared with sham animals (Chi et al. 2018). Furthermore, Beclin1 and Atg5 protein expression was significantly lower in the remote non-infarcted region 4 weeks post-MI compared with sham animals (Chi et al. 2018). Although statistically insignificant, p62 protein levels tended to increase 4 weeks post-MI compared with sham animals (Chi et al. 2018). On the other hand, it has also been shown that autophagic activity is augmented in the non-infarcted remote area and border area post-MI, and the autophagic activity progressively increases in the remote area up to 3 weeks post-MI (Kanamori, Takemura, Goto, Maruyama, Tsujimoto, et al. 2011; Kanamori et al. 2013). In the current study, we observed increased LC3-II protein levels and decreased p62 protein levels in WT non-infarct LV 28 days post-MI, suggesting autophagic alterations. Interestingly, ATM deficiency did not affect autophagic response in the non-infarct LV region 28 days post-MI. Thus, it is presently unclear why autophagic activity was unchanged in the non-infarct region of the heart 28 days post-MI in response to ATM deficiency and such a phenomenon warrant future investigations.

Collectively, these results indicate that ATM deficiency serves to enhance autophagy under basal conditions while also augmenting autophagic activity in the infarct region during a chromic model of MI (28 days post-MI) and 24 hours post ATM inhibition in cardiac fibroblasts. However, during the early stages of MI (4 hours during MI) and 4 hours following ATM inhibition in cardiac fibroblasts, ATM deficiency impairs autophagic response. While autophagic enhancement during ATM deficiency is mTOR dependent under basal conditions, its augmentation is mTOR independent in the infarct region 28 days post-MI. Furthermore, autophagic response during ATM deficiency in the heart and in cardiac fibroblasts does not seem

to involve other signaling molecules (Akt, GSK-3β, Erk1/2, AMPK) until the onset of MI. Four hours after the onset of MI, autophagic impairment is driven by inactivation of Akt and AMPK, and activation of GSK-3β and m-TOR. On the contrary, autophagic changes in the infarct region 28 days post-MI associates with activation of Akt, Erk1/2, and mTOR and inactivation of GSK-3β. These results suggest that the role of ATM in autophagic response changes over the time course of MI (ATM deficiency has different effects in autophagy 4 hours into MI as opposed to 28 days post-MI), and the mechanisms by which these changes occur with time are also different. Thus, future investigations involving intermediate time points between 4 hours and 28 days post-MI should be conducted to understand the full scope of molecular changes involved in autophagic flux during ATM deficiency post-MI. Also, the utilization of autophagy inhibitors and activators in future studies may help elucidate the mechanisms by which ATM deficiency alters autophagy post-MI.

## ATM and Cardiac Glucose Metabolism

Another interesting major finding of this investigation is that the lack of ATM accelerates glycolysis and gluconeogenesis, and augments TCA cycle metabolism under non-ischemic conditions. Additionally, ATP levels were lower in ATM null hearts under non-ischemia, while glycogen levels were comparable between WT and KO hearts. The enhanced glycolysis in ATM null hearts suggest metabolic stress. It is possible that glycolysis is enhanced in KO hearts as an attempt to replenish ATP levels and proper functioning of the heart. However, these efforts may somewhat be restrained by enhanced gluconeogenesis, another mode by which homeostasis could be restored (Aguiar et al. 2017). It is also possible that glycogen levels remained unchanged under non-ischemic conditions in KO hearts due to rapid cycling of both glycolytic

and gluconeogenic pathways. Furthermore, 20 minute global ischemia down-regulated the glycolytic pathway, not the gluconeogenic pathway, during ATM deficiency. Glycogen stores were also significantly reduced in ATM null hearts post-ischemia. Glycogen is a critical energy repository in the heart and can quickly be mobilized if needed (Wang et al. 2014). During energy deficiency, glycogen breakdown is enhanced to accommodate the rapid changes in cardiac demand (Wang et al. 2014). When coupled with decreased glycolysis, a decrease in glycogen levels indicate a preference for glycogen metabolism as opposed to metabolizing exogenous glucose in KO hearts post-ischemia.

AMPK is a key regulator of glucose metabolism as its activation triggers glycolysis to produce ATP (Long and Zierath 2006; Heidrich et al. 2010; Wu and Wei 2012). To increase glucose uptake, AMPK increases GLUT1 expression and GLUT4 translocation to the plasma membrane (Heidrich et al. 2010). Like AMPK, Akt promotes glucose uptake by increasing GLUT1 expression on the plasma membrane and promoting the translocation of GLUT4 to the plasma membrane (Chaanine and Hajjar 2011; Ward and Thompson 2012). Akt also phosphorylates or inactivates GSK-3β, which in turn stimulates glycogen synthesis (Oreña et al. 2000; Lee and Kim 2007). Here, activation of AMPK was higher in ATM null hearts under non-ischemic conditions, suggesting its involvement in the accelerated glycolysis in response to low ATP levels. However, global ischemia decreased AMPK activation in ATM null hearts, a phenomenon that may explain low GLUT4 protein expression and glycolysis in these hearts. Global ischemia enhanced GSK-3β activity, while decreasing Akt activity in KO hearts. These data suggest that glycogen consumption is regulated by Akt/GSK-3β pathway in KO hearts post-ischemia, and that Akt activity may also be involved in Glut4 expression as Glut4 expression was lower in those hearts post-ischemia as well.

ATM null hearts exhibited altered TCA cycle metabolism under non-ischemic conditions. Isocitrate levels were lower, while α-ketoglutarate, succinate, and fumarate levels were higher in null hearts. The decrease in isocitrate levels could be due to the usage of pyruvate to fuel glycolytic and gluconeogenic cycling as opposed to being utilized to drive the TCA cycle. Succinate plays a critical role in the heart's response to ischemia-reperfusion injury and is considered a universal signature of ischemia (Chouchani et al. 2014; Pell et al. 2016). Accumulation of fumarate is suggested to play a cardioprotective role (Ashrafian et al. 2012). The increase in the levels of α-ketoglutarate, succinate, and fumarate may in part be attributable to the contribution of amino acid metabolism. Global ischemia decreased citrate and α-ketoglutarate levels in null hearts, however, fumarate levels were increased, indicating that the TCA cycle may be defective or down-regulated in the absence of ATM during global ischemia. Fumarate levels are higher due to its potential cardio-protective effect.

Altogether, these results indicate that the lack of ATM results in accelerated gluconeogenesis and glycolysis under non-ischemic conditions and that global ischemia down-regulates glycolysis in ATM null hearts. Such changes were also accompanied by alterations in TCA cycle metabolism that may serve as cardio-protective. Overall changes in glucose metabolism in ATM null hearts were driven by alterations in the activity of AMPK, Akt, and GSK-3β as well as protein levels of Glut4. Although these findings are novel and hold the potential to shed light on the role of ATM in glucose metabolism, a thorough time course analysis of various metabolites and signaling molecules involved metabolism is needed to provide further insights into the mechanism by which ATM affects cardiac energetics.

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